U.S. DEPARTMENT OF COMMERCE
National Technical Information Service
PB-278 631

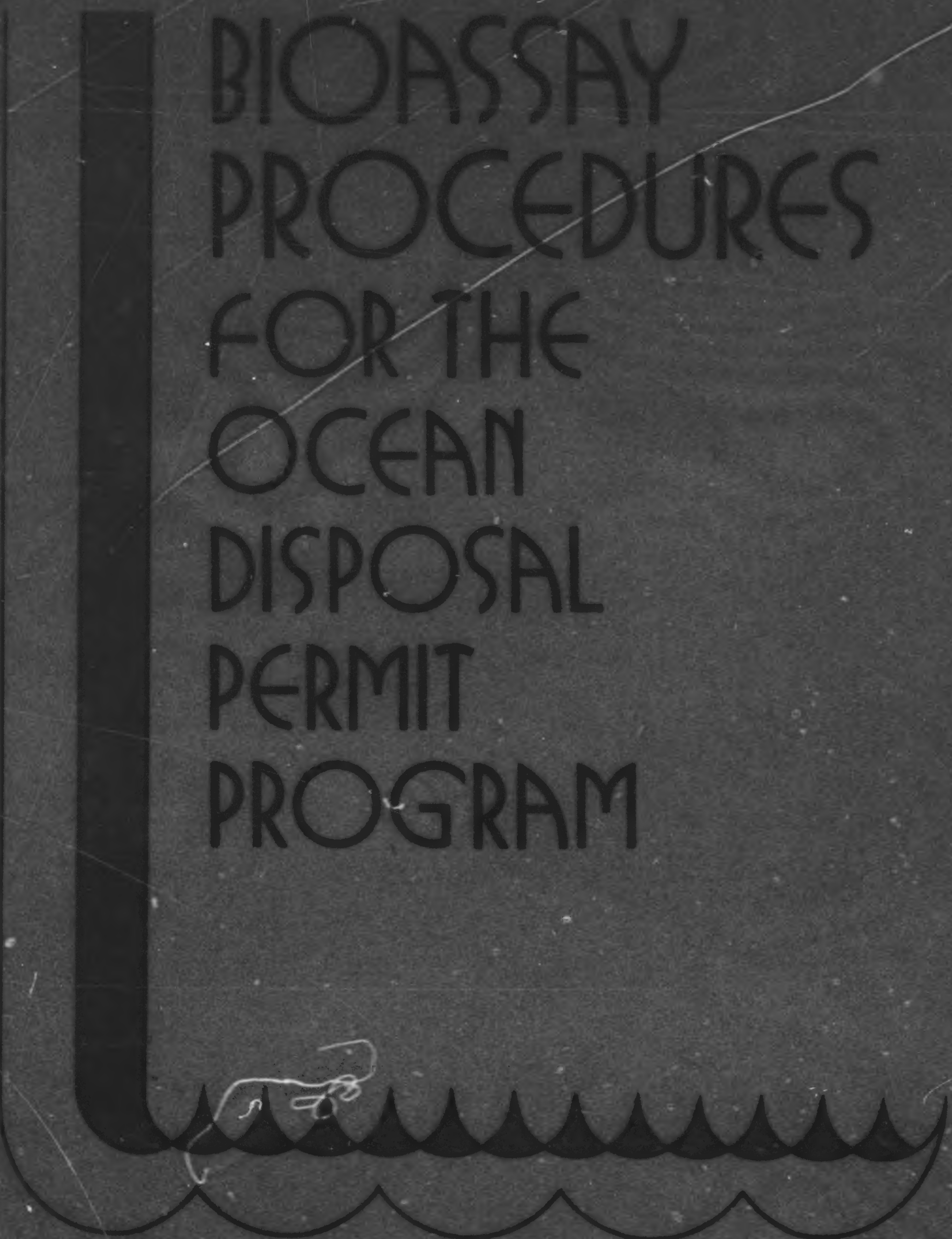
Bioassay Procedures for the Ocean Disposal Permit Program

Environmental Research Lab, Gulf Breeze, Fla

Mar 78

PB 278 631

EPA-600/9-78-010
MARCH 1978



BIOASSAY PROCEDURES FOR THE OCEAN DISPOSAL PERMIT PROGRAM

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
GULF BREEZE, FLORIDA 32561

134

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/9-78-010	2.	3. RECIPIENT'S ACCESSION NO. PB278631
4. TITLE AND SUBTITLE Bioassay Procedures for the Ocean Disposal Permit Program		5. REPORT DATE Issuing Date March 1978
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) ENVIRONMENTAL PORTECTION AGENCY OCEAN DISPOSAL BIOASSAY WORKING GROUP		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Environmental Protection Agency Ocean Disposal Bioassay Working Group Environmental Research Laboratory, Gulf Breeze, FL.		10. PROGRAM ELEMENT NO. 1EA714
		11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Gulf Breeze, Florida 32561		13. TYPE OF REPORT AND PERIOD COVERED In-house/Final
		14. SPONSORING AGENCY CODE EPA/600/08

15. SUPPLEMENTARY NOTES

16. ABSTRACT

The bioassay procedures given in this manual were developed to provide tests for conducting toxicity evaluations of waste materials considered for ocean disposal under EPA's Ocean Disposal Permit Program.

The procedures specify the use of various organisms representing several trophic levels. Both flow-through and static tests are included. Methods given vary in their utility and complexity of performance. The procedures are not intended to be considered "standard methods," but, depending on the judgment of the EPA Regional Administrator responsible for the managing of the permit program, are to be used as reference methods or official methods.

This manual is a revision of EPA-600/9-76-010 published May 1976.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Bioassay, Oysters, Marine fishes, Algae, Crustacea	Bioassay procedures, Ocean Disposal Permit Program, Marine organisms, Marine phytoplankton, Brine shrimp, Calanoid copepods, Macroinvertebrates, Fish brain acetylcholinesterase	6F 6T

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) Unclassified	21. NO OF PAGES 134
	20. SECURITY CLASS (This page) Unclassified	22. PRICE PC A07/A01

i

EPA-600/9-78-010
March 1978

BIOASSAY PROCEDURES FOR THE OCEAN DISPOSAL PERMIT PROGRAM

by

Environmental Protection Agency
Ocean Disposal Bioassay
Working Group

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL RESEARCH LABORATORY
GULF BREEZE, FLORIDA 32561

ia

DISCLAIMER

This report has been reviewed by the Environmental Research Laboratory, Gulf Breeze, U. S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

The Marine Protection, Research, and Sanctuaries Act of 1972, as amended, (MPRSA) requires that applications for permits for ocean dumping be evaluated on the basis of their ecological impact on the marine environment, as well as on other considerations included in the MPRSA. The International Convention on the Prevention of Marine Pollution from the Dumping of Wastes and Other Matter, (Convention) for which the MPRSA is the enabling domestic legislation, requires a similar evaluation and also prohibits the disposal of mercury and its compounds, cadmium and its compounds, organohalogens, and oils and greases as other than trace contaminants.

The revised EPA ocean dumping regulations and criteria (40 CFR, 220-228), published January 11, 1977, establish bioassays as the key procedures to be used to assess the probable ecological impact of particular wastes, and also establish criteria by which bioassay results are to be used to determine whether or not a waste is environmentally acceptable for ocean dumping.

Bioassay procedures described in this manual were developed for use by EPA personnel in carrying out the ocean dumping permit program under the MPRSA and pursuant to the revised EPA ocean dumping regulations and criteria. This manual is a revision of EPA-600/9-76-010 published May, 1976, and includes improvements in bioassay procedures which represent recent advances in the state-of-the-art in marine bioassay techniques. As such, they should be considered recommended interim procedures and not as definitive standard methods.

The procedures presented here cover a wide variety of techniques and organisms. Selection of appropriate procedures should be made by the permitting authority on a case-by-case basis, depending on the type and amount of waste, location of dump site, proposed methods of disposal, and other appropriate considerations.

Thomas W. Duke

Thomas W. Duke
Director
Environmental Research Laboratory
Gulf Breeze, Florida 32561

ABSTRACT

The bioassay procedures given in this manual were developed to provide tests for conducting toxicity evaluations of waste materials considered for ocean disposal under EPA's Ocean Disposal Permit Program.

The procedures specify the use of various organisms representing several trophic levels. Both flow-through and static tests are included. Methods given vary in their utility and complexity of performance. The procedures are not intended to be considered "standard methods," but, depending on the judgment of the EPA Regional Administrator responsible for the managing of the permit program, are to be used as reference methods or official methods.

This manual is a revision of EPA-600/9-76-010 published May 1976.

CONTENTS

Foreword iii

Abstract iv

Figures vii

Tables viii

Contributors ix

I. Introduction 1

II. Bioassay Procedures 2

- A. Background Information for the Performance of Phytoplankton Marine Bioassays 2
- B. Static Method for Acute Toxicity Tests with Phytoplankton 19
- C. Flowing Sea Water Toxicity Test Using Oysters (*Crassostrea virginica*) 25
- D. Methods for the Culture and Short-Term Bioassay of the Calanoid Copepod (*Acartia tonsa*). . 28
- E. Culturing the Mysid (*Mysidopsis bahia*) in Flowing Sea Water or a Static System 59
- F. Acute Static Toxicity Tests with Mysid Shrimp (*Mysidopsis bahia*) 61
- G. Entire Life-Cycle Toxicity Test Using Mysids (*Mysidopsis bahia*) in Flowing Water 64
- H. Culture of the Grass Shrimp (*Palaemonetes pugio*) in the Laboratory 69
- I. Static Bioassay Procedures Using Grass Shrimp (*Palaemonetes* sp.) Larvae 73
- J. Entire Life-Cycle Toxicity Test Using Grass Shrimp (*Palaemonetes pugio* Holthuis) 83

K. Static Method for Acute Toxicity Tests Using Fish and Macroinvertebrates 89

L. Flow-through Methods for Acute Toxicity Tests Using Fish and Macroinvertebrates 97

M. Laboratory Culture of Sheepshead Minnows (Cyprinodon variegatus) 107

N. Life-Cycle Toxicity Test Using Sheepshead Minnows (Cyprinodon variegatus) 109

O. Fish Brain Acetylcholinesterase Inhibition Assay 118

Appendices 43

1-D Synthetic Sea Water Formulation 43

2-D Sea Water and Sterility Enrichment 44

3-D Algal Culture 45

4-D Descriptive Characteristics for Selected Neritic Copepods 46

FIGURES

Number		Page
1-A	Hypothetical relationship between algal growth and toxicant concentration	10
2-A	Relationship between percentage of control growth rate (0-48 hrs) and copper	13
1-D	Mass Copepod Culture Systems (Static) . . .	32
2-D	Generation Cage	33
3-D	Mass Copepod Culture (Flowing).	35
4-D	Bioassay protocol	38
1-H	A flow-through hatching apparatus for grass shrimp larvae production	70
1-I	Example of a range-finding bioassay	74
2-I	Example of a definitive bioassay	75

TABLES

Number		Page
1-A	Sea water and sterility enrichments	4
2-A	Synthetic sea water formulation for algal assays	5
3-A	Nutrient enrichments for algal bioassay medium	7
1-B	Composition of mixes to be added to artificial sea water	21
1-D	Composition of algal diet and recommended concentration for feeding, egg laying, and naupliar feeding	29
2-D	Protocol for mass copepod culture	34
1-K	Standard salt water	90
2-K	Suggested sea water test temperatures for vertebrates and invertebrates	93
1-L	Maximum sea water test temperatures for vertebrates and invertebrates	102

Entire Life-Cycle Toxicity Test Using Grass Shrimp (*Palaemonetes pugio* Holthuis)
Dana Beth Tyler-Schroeder, Environmental Research Laboratory, Gulf Breeze, Florida

Static Method for Acute Toxicity Tests Using Fish and Macroinvertebrates
David J. Hansen, Steven C. Schimmel, Del Wayne Nimmo, and Jack I. Lowe, Environmental Research Laboratory, Gulf Breeze, Florida
Patrick R. Parrish, (formerly Environmental Research Laboratory, Gulf Breeze; now EG&G, Marine Research Laboratory, Pensacola, Florida)
William H. Peltier, EPA, Region IV, Atlanta, Georgia

Flow-through Methods for Acute Toxicity Tests Using Fish and Macroinvertebrates
David J. Hansen, Steven C. Schimmel, Del Wayne Nimmo, and Jack I. Lowe, Environmental Research Laboratory, Gulf Breeze, Florida
Patrick R. Parrish, (formerly Environmental Research Laboratory, Gulf Breeze; now EG&G, Marine Research Laboratory, Pensacola, Florida)
William H. Peltier, EPA, Region IV, Atlanta, Georgia

Laboratory Culture of Sheepshead Minnows (*Cyprinodon variegatus*)
D. J. Hansen, Environmental Research Laboratory, Gulf Breeze, Florida

Life-Cycle Toxicity Test Using Sheepshead Minnows (*Cyprinodon variegatus*)
D. J. Hansen, S. C. Schimmel, and L. R. Goodman, Environmental Research Laboratory, Gulf Breeze, Florida
Patrick R. Parrish, (formerly Environmental Research Laboratory, Gulf Breeze; now EG&G, Marine Research Laboratory, Pensacola, Florida)

Fish Brain Acetylcholinesterase Inhibition Assay
David L. Coppage, Environmental Protection Agency, Washington, D. C.

Members of the EPA Ocean Disposal Bioassay Working Group are:
Thomas W. Duke, Chairman, Office of Research and Development (ORD), Environmental Research Laboratory, Gulf Breeze, Florida
William P. Davis, ORD, Environmental Research Laboratory, Gulf Breeze, Florida; Bears Bluff Field Station, South Carolina
Jack Gentile, ORD, Environmental Research Laboratory, Narragansett, Rhode Island
David J. Hansen, and Jack I. Lowe, ORD, Environmental Research Laboratory, Gulf Breeze, Florida
William E. Miller, ORD, Environmental Research Laboratory, Corvallis, Oregon
Royal J. Nadeau, Region II, Edison, New Jersey
Carolyn K. Offutt, Office of Water and Hazardous Material (OWHM), Washington, D. C.
Richard D. Spear, Region II, Edison, New Jersey

CONTRIBUTORS

Bioassay procedures published in this manual resulted from deliberations of the Ocean Dumping Bioassay Committee and represent methodology developed at EPA laboratories. Contributing laboratories and investigators follow:

Static Method for the Performance of Phytoplankton
John H. Gentile and Mimi Johnson, Environmental Research Laboratory, Narragansett, Rhode Island

Flowing Sea Water Toxicity Test Using Oysters (Crassostrea virginica)
Phillip A. Butler, Office of Pesticide Programs at Environmental Research Laboratory, Gulf Breeze, Florida
Jack I. Lowe, Environmental Research Laboratory, Gulf Breeze, Florida

Methods for the Culture and Short-Term Bioassay of the Calanoid Copepod (Acartia tonsa)
John H. Gentile and Suzanne Sosnowski, Environmental Research Laboratory, Narragansett, Rhode Island

Culturing the Mysid (Mysidopsis bahia) in Flowing Sea Water or a Static System
D. R. Nimmo, T. L. Hamaker, and C. A. Sommers, Environmental Research Laboratory, Gulf Breeze, Florida

Acute Static Toxicity Tests with Mysid Shrimp (Mysidopsis bahia)
Patrick W. Borthwick, Environmental Research Laboratory, Gulf Breeze, Florida

Entire Life-Cycle Toxicity Test Using Mysids (Mysidopsis bahia) in Flowing Water
D. R. Nimmo, T. L. Hamaker, and C. A.Sommers, Environmental Research Laboratory, Gulf Breeze, Florida

Culture of the Grass Shrimp (Palaemonetes pugio) in the Laboratory
Dana Beth Tyler-Schroeder, Environmental Research Laboratory, Gulf Breeze, Florida

Static Bioassay Procedures Using Grass Shrimp (Palaemonetes sp.) Larvae
Dana Beth Tyler-Schroeder, Environmental Research Laboratory, Gulf Breeze, Florida

Entire Life-Cycle Toxicity Test Using Grass Shrimp (*Palaemonetes pugio* Holthuis)

Dana Beth Tyler-Schroeder, Environmental Research Laboratory, Gulf Breeze, Florida

Static Method for Acute Toxicity Tests Using Fish and Macroinvertebrates

David J. Hansen, Steven C. Schimmel, Del Wayne Nimmo, and Jack I. Lowe, Environmental Research Laboratory, Gulf Breeze, Florida

Patrick R. Parrish, (formerly Environmental Research Laboratory, Gulf Breeze; now EG&G, Marine Research Laboratory, Pensacola, Florida)

William H. Peltier, EPA, Region IV, Atlanta, Georgia

Flow-through Methods for Acute Toxicity Tests Using Fish and Macroinvertebrates

David J. Hansen, Steven C. Schimmel, Del Wayne Nimmo, and Jack I. Lowe, Environmental Research Laboratory, Gulf Breeze, Florida

Patrick R. Parrish, (formerly Environmental Research Laboratory, Gulf Breeze; now EG&G, Marine Research Laboratory, Pensacola, Florida)

William H. Peltier, EPA, Region IV, Atlanta, Georgia

Laboratory Culture of Sheepshead Minnows (*Cyprinodon variegatus*)

D. J. Hansen, Environmental Research Laboratory, Gulf Breeze, Florida

Life-Cycle Toxicity Test Using Sheepshead Minnows (*Cyprinodon variegatus*)

D. J. Hansen, S. C. Schimmel, and L. R. Goodman, Environmental Research Laboratory, Gulf Breeze, Florida

Patrick R. Parrish, (formerly Environmental Research Laboratory, Gulf Breeze; now EG&G, Marine Research Laboratory, Pensacola, Florida)

Fish Brain Acetylcholinesterase Inhibition Assay

David L. Coppage, Environmental Protection Agency, Washington, D. C.

Members of the EPA Ocean Disposal Bioassay Working Group are:

Thomas W. Duke, Chairman, Office of Research and Development (ORD), Environmental Research Laboratory, Gulf Breeze, Florida

William P. Davis, ORD, Environmental Research Laboratory, Gulf Breeze, Florida; Bears Bluff Field Station, South Carolina

Jack Gentile, ORD, Environmental Research Laboratory, Narragansett, Rhode Island

David J. Hansen, and Jack I. Lowe, ORD, Environmental Research Laboratory, Gulf Breeze, Florida

William E. Miller, ORD, Environmental Research Laboratory, Corvallis, Oregon

Royal J. Nadeau, Region II, Edison, New Jersey

Carolyn K. Offutt, Office of Water and Hazardous Material (OWHM), Washington, D. C.

Richard D. Spear, Region II, Edison, New Jersey

SECTION 1

INTRODUCTION

The bioassays procedures given in this manual were established to provide procedures for conducting biological evaluation of waste materials to be disposed of in the ocean. Tests conducted according to these bioassay procedures will provide information on the toxicity of various materials to be disposed. However, these bioassay procedures, like all laboratory bioassay methods, are attempts at simulation of actual conditions and therefore suffer all the inaccuracies inherent to simulation systems.

Although these bioassay procedures are not "standard" EPA methods, they are intended as guides for those involved in evaluating ocean dumping permits. Accordingly, each method differs in detail and style and does not conform to a standard format. Permit applicants are expected to modify bioassay procedures according to both the nature of the waste material and the type of procedure involved.

The Ocean Dumping Bioassay Committee requires that a minimum of three species be used in an evaluation of a permit. These species should be selected from the different taxonomic groups listed in the section on the flow-through method for acute toxicity tests using fish and macroinvertebrates (see page 97). We recommend that indigenous organisms be used whenever possible in addition to those organisms recommended in this manual.

The EPA bioassay working group intends to revise these bioassay procedures periodically as new information becomes available. We are coordinating our efforts with the EPA/Corps of Engineers Technical Committee on Criteria for Dredge and Fill Committee.

SECTION II

BIOASSAY PROCEDURES FOR ROUTINE APPLICATION

A. BACKGROUND INFORMATION FOR THE PERFORMANCE OF PHYTOPLANKTON MARINE BIOASSAYS

The primary producer populations of estuaries consist principally of microscopic phytoplankton. In their role of storing potential energy, via photosynthesis, these organisms represent the primary energy input into aquatic ecosystems (Joint Industry/Government Task Force, 1969). Thus, it is imperative that water quality conditions be favorable to their growth and reproduction if serious alterations in other components of marine communities are to be avoided.

Under natural conditions, both the qualitative and quantitative aspects of phytoplankton population dynamics display a high degree of seasonability, characterized by well-defined succession patterns. It is essential that not only the productivity of various systems be maintained, but also the relative abundance of species according to normal seasonal compositions because primary herbivore populations exhibit selectivity in their grazing patterns. Consequently, while a pollutant may seem to have no apparent adverse effect on the total primary production, it may have drastically altered community structure and composition. Such alterations often occur when sensitive indigenous species are replaced by species less desirable ecologically, but equally active photosynthetically. If the more resistant species is incompatible with the feeding and/or nutritional requirements of primary herbivore populations, then energy transfer to high trophic levels will be affected and contribute ultimately to significant effects on naturally occurring desirable populations. Data for the phytoplankton are a necessity to adequately describe and predict the potential effects of a toxicant upon an estuarine ecosystem response.

1. Species Selection

In the design of a bioassay program, the selection of test species is pivotal to the acquisition of realistic and meaningful information. Algal culture techniques historically have focused upon developing suitable culture media to sustain complete life cycles. Nutritional levels and medium composition often bore little resemblance to the actual environmental conditions the organism encountered. Furthermore, research was often limited to a few species that were readily maintained in the laboratory.

Within the last decade, culture techniques have greatly broadened the scope of species available for investigation.

In choosing species for bioassays, the following criteria are useful guides:

a. Whenever possible, indigenous species representing a diversity of phylogenetic types from the major seasonal successions should be studied.

b. Since sensitivities vary among species, when possible, more sensitive species should be selected for bioassay.

c. From seasonal and laboratory studies, conditions of greatest vulnerabilities should also be identified for the species selected.

d. Since a bioassay basically measures the response of an organism to the product of toxicant concentration and exposure time, the rate of response of the test species must be considered. Both test species and culture conditions should permit growth rates of 0.5-1.0 doublings per day under non-stress conditions.

The above criteria offer maximum flexibility for the experienced researcher. For workers with limited funds and expertise, two species are recommended if indigenous forms are unavailable: Skeletonema costatum (species of choice) is an ecologically important phytoplankter that is common to a wide geographic range of neritic waters. Thalassiosira pseudonana, while of lesser ecological significance, is sensitive to heavy metals and has an 8-hour generation time which offers greater practical value in the establishment of toxicological responses. It is also recommended that these species be used in conjunction with others to serve as controls on the systems being tested.

2. Culture Conditions

The culture conditions for all test species generally should reflect their natural conditions. In order to develop some semblance of uniformity, two basic regimes are recommended. For temperate species, a temperature of 20° ± 2°C, 2500-5000 lux on a 14-hour light and 10-hour dark cycle (14:10 cycle) is desirable. For cold water forms, a temperature of 8° ± 2°C, 2500-5000 lux on a 10:14 cycle is recommended. Stock cultures of the test species are to be maintained in enriched natural (Table 1-A), or synthetic sea waters (Table 2-A).

The stock cultures should be transferred to the nutritionally dilute culture medium and allowed to complete two growth cycles prior to use in a bioassay. This is necessary since nutritional history can have marked effects upon responses. We have found up to five-fold differences in responses of bioassay organisms maintained under high and natural nutrient levels (Gentile et al., 1973). Stock cultures should be maintained with sterile transfer techniques.

TABLE 1-A. SEA WATER AND STERILITY ENRICHMENTS

Sea water enrichments for stock algal culture maintenance (After Guillard and Ryther, 1962):

$NaNO_3$	75 mg/liter
$NaH_2PO_4.H_2O$	5 mg/ℓ
$Na_2SiO_3.9H_2O$	10 mg/ℓ
Vitamins:	
Thiamine HCl	0.10 mg/ℓ
Biotin	0.50 μg/ℓ
B_{12}	0.50 μg/ℓ
Trace metals:	
$CuSO_4 5H_2O$	0.002 mg/ℓ
$ZnSO_4 5H_2O$	0.004 mg/ℓ
$CoCl_2.6H_2O$	0.002 mg/ℓ
$MnCl_2.4H_2O$	0.036 mg/ℓ
$NaMoO_4.2H_2O$	0.001 mg/ℓ
Fe-sequestrine	1.0 mg (0.13 mg Fe)/ℓ

Buffer:

TRIS-500 mg/ℓ @ pH 7.8-8.2

Before autoclaving, the following sterility enrichments should be added to the enriched sea water medium above:

Sodium glutamate	250 mg/ℓ
Sodium acetate	250 mg/ℓ
Cycline	250 mg/ℓ
Nutrient agar	50 mg/ℓ
Sucrose	250 mg/ℓ
Sodium lactate	250 mg/ℓ
L & D alanine	250 mg/ℓ

TABLE 2-A. SYNTHETIC SEA WATER FORMULATION FOR ALGAL ASSAYS*

Compound	Concentration/liter
NaCl	24.00 g
Na_2SO_4	4.00 g
H_3BO_3	0.03 g
$CaCl_2 . 2H_2O$	1.47 g
$MgCl_2 . 6H_2O$	10.78 g
$Na_2Si{:}O_3.9H_2O$ †	30.00 mg
KCl	700.00 mg
$NaHCO_3$	200.00 mg

*Adapted from original table, Kester et al., 1967.
†Prepare stock solution in deionized water and adjust to pH 7.8-8.2

3. Sea Water

The choice of sea water is dictated by availability, quality, and cost. Natural sea water can often be used for bioassays even though inherent variables in quality may complicate analysis of results. Clean offshore water is suitable if proper precautions are observed during collection and processing. In general, synthetic sea water is preferred for the constancy of its composition and quality even though trace contaminants must be removed by additional purification. The cost of the required chemicals and purification is usually equivalent to the expense of collecting, transporting, and processing natural sea water.

a. Natural Sea Water

Sea water is collected from 3-10 meters below the surface (to avoid surface contamination) with a non-metallic water sampler, and transported in autoclavable polyethylene carboys. Glass is also suitable if breakage can be prevented. Within 24 hours, the water is filtered through acid-washed membrane filters in a non-metallic filtration system. Filtered sea water is then stored at 4°C in the dark.

b. Synthetic Sea Water

Commercially available synthetic salt water is also acceptable; when heavy metal toxicity is being tested, an iron-chelate version should be employed. It is suggested that these sea water mixtures be aged and aerated for 24 hours before use.

A modified synthetic sea water formulation (Table 2-A) has been developed from Kester et al. (1967). This sea water is recommended for fish, invertebrates, and plankton bioassays. This synthetic sea water has been endorsed by the Environmental Protection Agency, the 14th Edition of Standard Methods, and the A.S.T.M. Committee on Bioassays.

c. Salinity

A salinity of 30 °/oo is recommended for all bioassays. Salinity adjustments on natural or synthetic sea waters should be made with glass distilled or deionized water.

d. Sterilization

Sterilization of stock culture maintenance medium can be satisfactorily achieved by autoclaving since the pH is stabilized by the presence of TRIS-buffer. Since bioassay medium cannot be autoclaved, two alternative methods are recommended: 1) positive pressure filtration and/or 2) pasteurization (60° ± 2° C for 4 hours). These treatments will not appreciably alter the physico-chemical properties of the sea water but will provide effective sterilization.

The medium, however, should be filtered (0.45μ) through a previously acid washed (2 N HCl) filter. Removal of residual acid is accomplished by rinsing the filter with distilled/deionized water and discarding the first liter of filtered sea water. Medium should be stored in acid-stripped borosilicate glass or linear polyethylene carboys, to which a sterile dispensing tower can be connected to distribute media.

Sterility checks are made weekly on this test medium by inoculating 2 ml aliquot of sea water into 10 ml of sterile water enriched as in Table 3-A. The tubes are incubated at 20°C in the dark for up to one week. Contamination is indicated by turbidity and opalescence of the medium.

TABLE 3-A. NUTRIENT ENRICHMENTS FOR ALGAL BIOASSAY MEDIUM

Nutrient	Amount
Na NO_3	4.42 mg/ℓ (50 μMN)
K_2HPO_4	0.87 mg/ℓ (5 μMP)
Thiamine	100.00 g/ℓ
Biotin	0.50 g/ℓ
B_{12}	0.50 g/ℓ
Fe*	25.00 g/ℓ
Mn	10.00 g/ℓ
Zn	1.00 g/ℓ
Mo	0.50 g/ℓ
Co	0.10 g/ℓ
Cu	0.10 g/ℓ

*Fe as Cl: Dissolve iron sponge or filings in minimum HCl with warming and dilute to volume with deionized water.

4. Glassware

All glassware is high grade borosilicate glass (Pyrex/Kimax). The bioassays, performed in 125-ml Erlenmyer flasks containing 50 ml of medium, are sealed with foam plugs. Glassware is dry-heat sterilized (170°C for 2 hours) rather than autoclaved, since the steam often carries metal contaminants which can interfere with bioassays involving metal toxicity.

Rigorous cleaning is necessary for all glassware to insure against contamination. Glassware is soaked in detergent, hand or mechanically brushed, rinsed in deionized water, totally immersed in 10% HNO_3 for 2-6 hours, thoroughly rinsed in double glass distilled or deionized water, and air or oven dried.

For work involving the toxicity of metals, the glassware should receive the following post-wash treatment: To eliminate the problems of either positive or negative contamination, a monolayer of silico-polymer is applied to all surfaces contacting the sea water. Commercially available SC-87* prepared as 5% solution in cyclohexane, is poured into the glassware and drained, leaving a film on the surface. The glassware is then air-dried and oven-cured at 150-175°C for 4 hours. The result is a completely non-wettable surface which, after a double glass distilled water rinse, is ready to use. One coating often lasts two or three assays before recoating is necessary. Recoating can be done over the old coating or a strong alkali (2N NAOH + 10% ETOH) can completely strip the old coating prior to recoating. In most instances, alcoholic-alkali stripping can be avoided by using hot detergent each time prior to recoating.

5. Bioassay Protocol

The bioassay design consists of three major integrated components: preparation of log-phase inoculum, nutrient enriched bioassay medium, and toxicant solutions.

a. Inoculum

Inoculum for the bioassay is prepared by inoculating 0.5 ml (0.1-1.0 ml) of stock culture into triplicate 125-ml flasks containing nutrient enriched sea water at bioassay level (Table 3-A). At the point of inflection of the growth curve, inoculate three new flasks from this series and follow the second growth curve. Cells from this second or later transfers are suitable for use in the bioassay. These cells now have adapted to the more natural nutrient levels, and their response will more closely reflect that expected from a natural population of the test species.

b. Bioassay Medium

Filter sterilized and/or pasteurized, enriched sea water is dispensed into a presterilized 1-2 liter flask that is compatible with a 50-ml Ace-dispenser (Cat. no. 8004, Ace Glass Co., Vineland, N.J.). Nutrients (Table 3-A) are aseptically added and inoculum (as described above) is added to give an initial cell density of 2,500 cells/ml to 10,000 cells/ml. Inoculation of the total medium volume permits the dispensing of a uniform cell population in all flasks. Initial cell density or biomass is measured. Fifty milliliters of enriched inoculated medium are dispensed into 125-ml flasks, using a 50 ml Ace-dispenser in a sterile hood.

---

*SC-87. Product of General Electric: distributed by Pierce Chemical Co., Rockford, Illinois.

The selection of an initial cell density will be dependent upon the sensitivity of the biomass parameter measuring system. For example, in clean systems using particle counters, initial cell densities of 2.5×10^3 microscopic counts are employed; initial cell densities of 1×10^4 cells/ml may be appropriate. For extractive (ATP, Chl "a") or isotope techniques, the initial cell density can be kept low since the aliquot examined can be adjusted.

c. Toxicant

Toxicant solutions are prepared in distilled water or suitable solvent for hydrophobic compounds. Stock solutions or dilutions of a waste should be prepared to ensure that the same volume is added at all test levels. This addition should not exceed one milliliter/50 ml of test medium. When working with waste effluents, a maximum of 5 ml addition is allowed since this will constitute a 10% maximum alteration in salinity. Toxicant additions are made to the flasks containing inoculated enriched sea water and placed in an incubator.

d. Design

The bioassay design is in part determined by the type of toxicant tested. A general format will include a screening of a broad range of concentrations from which levels are selected for a definitive evaluation. Generally, preliminary screenings should cover concentrations at four orders of magnitude with duplicate cultures at each level. The definitive assay should include one concentration above and two below the calculated 50% inhibition level, using logarithmic bisection of intervals. Triplicate cultures should be used for the definitive bioassay.

Parameter measurement should be evaluated at least once every 24 hours for the duration of the experiment. This permits calculation of rates of response which are important in interpreting the behavior of the toxicant. The duration of the experiment should be adequate for the control population to complete its logarithmic growth phase and reach a stationary growth rate. It is also desirable to determine for the inhibited cultures: the duration of the lag-phase, maximum rate of growth, and maximum yield (Figure 1-A). However, not all this information may be readily available from a single assay and all concentrations.

e. Modifications

The assay system described above uses small volumes (50 ml/125). This is not meant to frustrate the expansion of assay volumes. The systems can be easily scaled up to the following dimensions of 125/250; 250/500; 500/1,000. With larger volume systems media, dispensing can be made directly into the sterile flasks. Nutrients and test species can also be added to each flask. This increases the potential for variability and contamination but, with experience, difficulties can be minimized. The larger systems require more assay medium and space. However, greater volumes will permit more frequent analysis of a greater number of parameters. This

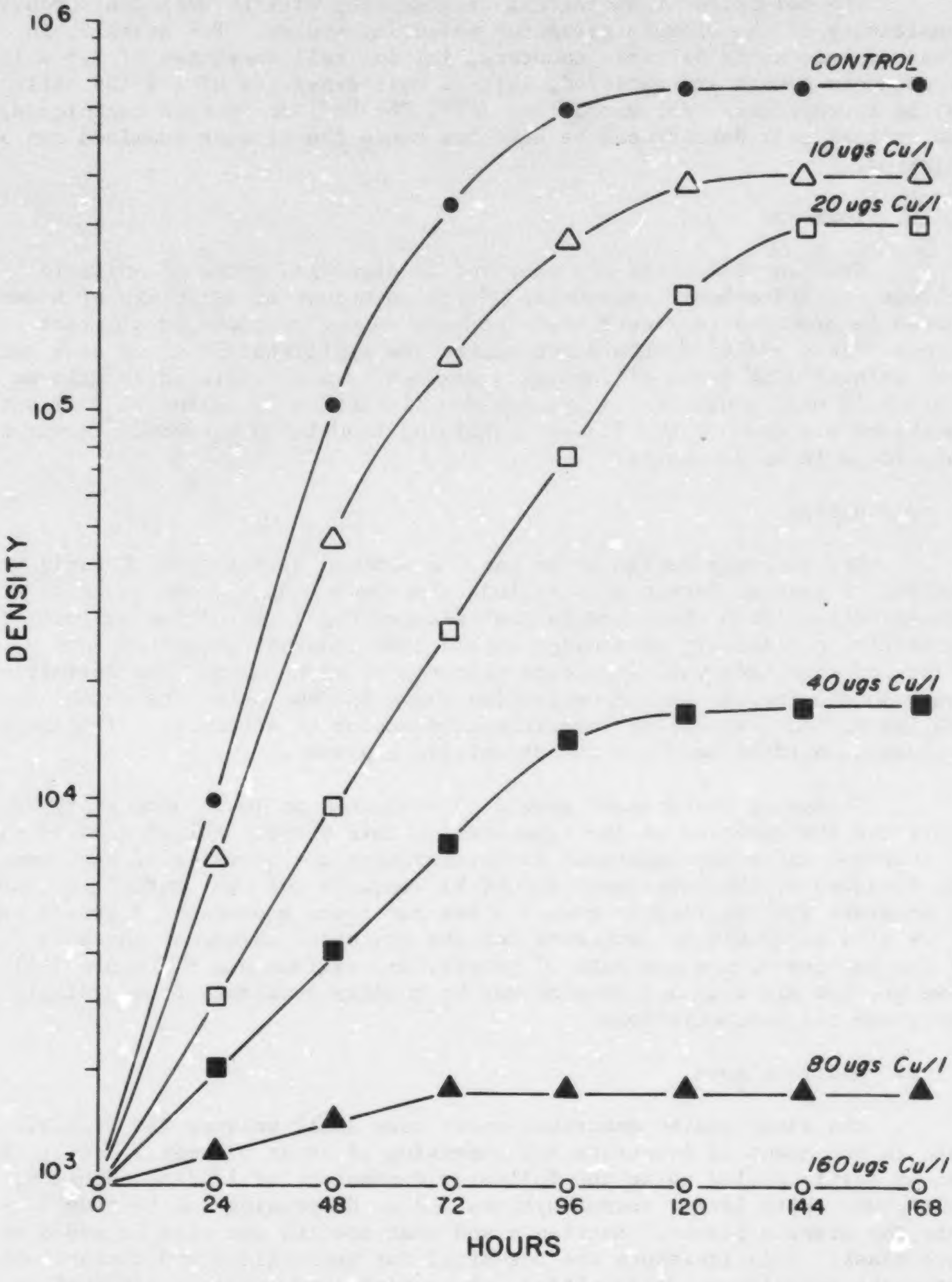


Figure 1-A. Hypothetical relationship between algal growth and toxicant concentration.

allows a more precise characterization of the anomalies resulting from specific pollutant exposures.

6. Parameters

There are a variety of parameters available that characterize the response of the algal cultures. These parameters are measures or indices of biomass at the time of sampling, which, when plotted against time, produce a growth response curve. This curve can be used to determine log-phase, rate of log-growth, and a maximum population density for control and exposed cultures.

a. Population Density

Microscopic measurements of cell density can be made using a haemocytometer, Palmer-Maloney Chamber, or inverted microscope with settling chambers. Details of these counting methods are available in the literature (Schwoerbel, 1970; American Public Health Association, 1975).

The microscopic methods present two problems: they are time-consuming when done properly and their statistical significance decrease significantly at cell densities below 1 x 10^4. Consequently, when large numbers of assays and replicates are required, it becomes impractical to count each assay microspically.

An electronic particle counter offers the most rapid, practical, and statistically accurate measurement of population density. The initial cost, while high, is offset by the increased work volume, accuracy, and saving of time.

b. Population Biomass

Biomass values can be calculated from population density data by using cell dimensions and assuming the cell is a particular geometrical shape (i.e., sphere, cylinder, etc.). This method, which depends on cell counts, is subject to the same limitations mentioned above.

Electronic particle counters can also give volumetric measurements, but usually such capabilities are obtained at additional cost. It is worth the expense if large numbers of assays are anticipated.

c. Chlorophyl

Chlorophyl "a" is often used as a measure of algal biomass. Both spectrophotometric absorbance and fluorescence (*in vivo* and *in vitro*) techniques are available (Strickland and Parsons, 1968). The spectrophotometric technique lacks the sensitivity particularly at low cell densities. The fluorescent systems, however, are more sensitive and can be used at cell densities of less than 1 x 10^4 cells/ml. The *in vivo* fluorescent technique is particularly useful because it does not require extraction and is very sensitive.

A potential limitation of this measurement is the general variability of cellular chlorophyl "a" as a function of nutrition and environmental variables (Odum et al., 1959; Yentsch and Ryther, 1957; Yentsch and Menzel, 1963).

d. Carbon-14 Assimilation

Productivity measurements, based upon radioactive carbon assimilation, is a standard technique applicable to both fresh water and marine algae (Steeman-Neilson, 1952; McAllister, 1961; Jitts, 1963; Jenkins, 1965; Strickland and Parsons, 1968). This is usually used as a short-term measure of photosynthetic activity. Culture aliquots may be pulse-labeled for four hours and C-14 incorporated by cells measured. This relative value may be used as a biomass index. This latter approach has shown a correlation to growth rates as measured by changes in cell number or biomass. Transient changes in C-14 assimilation, not reflecting long-term growth responses, have also been noted and warrant cautious interpretation of these data.

Adequate C-14 counting procedures may be obtained in Brandsom (1970) and Chase and Rabinowitz (1967).

e. ATP-Concentration

ATP has been suggested as a sensitive and accurate measure of living biomass due to a constancy of cellular ATP/carbon ratio (Holm-Hansen and Booth, 1966; Hamilton and Holm-Hansen, 1967; Holm-Hansen, 1969). Studies have demonstrated excellent correlation between ATP and direct measures of biomass (particle counting) and pulse labeling with carbon-14 (Gentile et al., 1973; Cheer et al., 1974). This technique requires instrumentation (about $5,000) and costs about $1.00 per analysis. As a measure of living material, highly contaminated wastes (i.e., sludge) could provide excessive interference.

The above techniques all offer certain advantages or disadvantages, depending on the bioassay design, type of effluent tested, facilities, and personnel.

Automated particle counting, while offering the most rapid, sensitive and statistically valid method, has limitations. The most restrictive relates to particle interferences. The test compound or effluent must have low background in the particle size range of the test species or inevitable masking and errors will result. This limits the types of effluents to be evaluated by this technique, unless the particulate fraction can be removed without jeopardizing the toxic characteristics of the material.

The other methods work well in systems containing particulate material, but both chlorophyl "a" and carbon-uptake have potentially undesirable response patterns that can make data interpretation difficult.

ATP, on the other hand, appears to be an excellent indicator of living biomass though it is somewhat expensive to measure routinely and may not be appropriate for biologically contaminated wastes (i.e., sludge).

All data can be converted to percentage control for any finite exposure period and the percentage response plotted versus toxicant concentration (Figure 2-A). From this graph, the relationship between toxicant concentration and degree of inhibition can be determined.

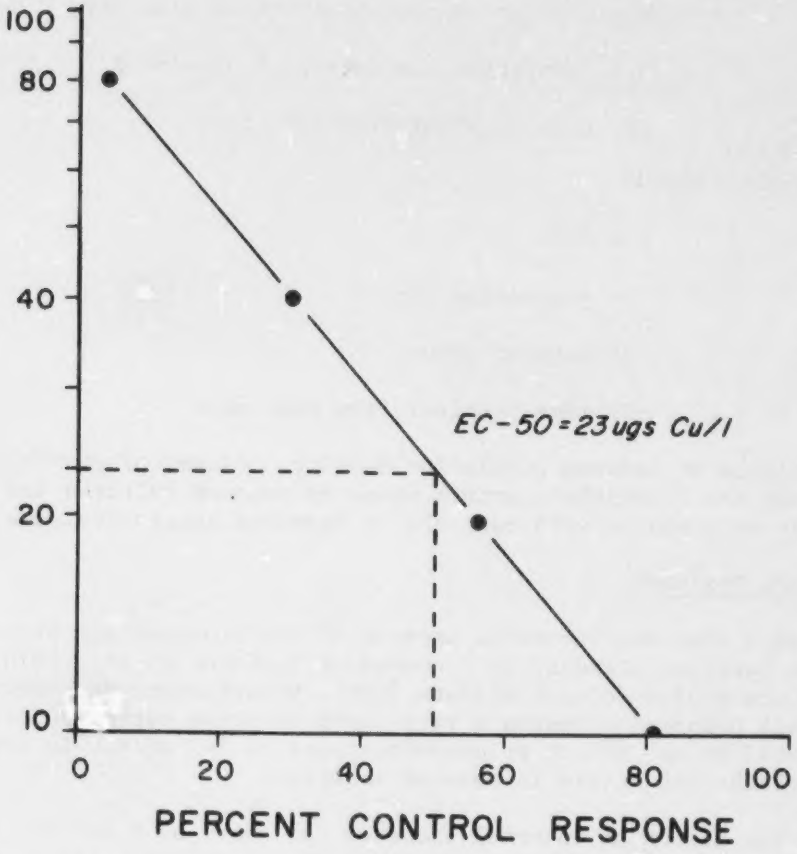


Figure 2-A. Relationship between percentage of control growth rate (0-48 hrs) and copper.

7. Data Presentation

The design of the bioassay requires a minimum of one observation every twenty-four hours for the duration of the experiment. Within this schedule, various options are available to the researcher. The basic data output represents a growth curve for all concentration examined. This may provide rate of growth:

$$k = \ell n \frac{N_t}{N_o} \; / \; \Delta T$$

k: rate of growth

N_o: population concentration at time zero

N_t: population concentration at time t

ΔT: time interval from time zero

and generation time:

$$G = \frac{\Delta T}{k}$$

G: generation time

k: rate of growth

ΔT: time interval from time zero

and comparisons at maximum population density. Slopes of growth curves representing the logarithmic growth phase of exposed cultures and population biomass may be compared with controls by standard statistical analysis.

8. Standard Toxicant

To insure that the technical aspects of the bioassay are properly performed, an internal standard is recommended (LaRoche et al., 1970). We routinely use sodium dodecyl sulfate (SDS), a surfactant and membrane lytic agent. This compound produces a very sharp response curve indicating an almost "total or no" effect at concentrations of 1-2 mg/ℓ. In addition, SDS is both soluble and stable in aqueous solutions.

While the use of an internal standard can serve as a quality assurance monitor, it does not, in itself, validate an experiment. There can be situations where the EC50 concentration for the standard toxicants in two experiments are essentially identical, but the control growth rates differ by a factor of two. The deviation of control growth from normal is an indication of a problem and thus warrants the repetition of the experiment. In addition, it is recommended that regional offices maintain a Quality Control Program by requiring contractors to process "blind" reference samples.

9. Applications

Algal bioassays, with their sensitivity and rapid response, are useful in many areas of water quality research.

a. The simplest application is for routine screening of potential toxicants. This represents a well-defined and controlled system where particle counting is recommended since interferences can usually be minimized. These studies should be designed to produce complete growth curves with both growth rate and maximum density output.

b. Another application of the algal bioassay is as an evaluation of water quality. If an impacted area is being investigated, water samples can be collected along a transect or matrix, depending on hydrographic data. The water is collected and processed according to techniques described in Section 4, and then inoculated with the test species that has been cultured in enriched water from a control station. Growth rate and population density can then be compared from station-to-station.

c. The algal assay can also be used to measure the biological impact of mixed effluents containing suspended solids. In this case, particle counting may not be practicable due to high levels of interference. Consequently, the growth of the algal culture can be monitored by obtaining daily aliquots and evaluating the ATP, chlorophyl "a," or measuring C-14 incorporation after pulse labeling the aliquot (2-4 hours) with $NaH^{14}CO_3$. The resulting data, when plotted semi-logarithmically with time, will produce a growth response curve that may be submitted to the interpretation discussed here.

d. Mention must be made of *in situ* applications of phytoplankton bioassay. Using ATP, C-14 uptake, and chlorophyl "a", both living biomass and productivity of a water mass may be estimated *in situ*. These studies can be made at the site; the samples are preserved and analyzed at a later date. Such applications, as evaluation of power plant entrainment and point-source pollution monitoring, commonly use this approach.

10. Remarks

It should be stressed that important advances have been made by the utilization of phytoplankton bioassays in the establishment of realistic water quality criteria for marine life.

Fundamental biological anomalies in phytoplankton could impair survival of high trophic levels and be associated with specific pollutant exposures. However, it should be noted that problems exist in the application of laboratory findings to conditions which may be found in the natural environment. One scientific discipline greatly neglected in this area is phytoplankton systematics. As a consequence, in many instances of *in situ* evaluation of phytoplankton productivity, identification of species will reveal the importance of knowing the species present.

REFERENCES

The following literature is recommended to the researcher for detailed discussions of techniques described in the text. It is not an exhaustive list but is adequate to acquaint the researcher with the analytical methodologies required to perform the assay successfully.

American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater, 14th Ed., 1027 pp.

Bransome, E.D., Jr. (Ed.) 1970. The Current Status of Liquid Scintillation Counting. Grune and Stratton, Inc., New York, 394 pp.

Chase, G.D., and J.L. Rabinowitz. 1967. Principles of Radioisotope Methodology, 3rd Ed. Burgess Publ. Co., Minneapolis, 633 pp.

Cheer, Sue, J. H. Gentile, and C. S. Hegre. 1974. Improved Methods for ATP Analysis. Analytical Biochemistry. 60:102-114.

Davey, E.W., J.H. Gentile, S.J. Erickson and P. Betzer. 1970. Removal of Trace Metals from Marine Culture Medium. Limnol. and Oceanogr. 15:486-488.

Gentile, J.H., S. Cheer, and P. Rogerson. 1973. The Effects of Heavy Metal Stress on Various Biological Parameters in Thalassiosira pseudonana. Abstract 34th Annual Meeting, Am. Soc. Limnol. and Oceanogr.

Hamilton, R.D., and O. Holm-Hansen. 1967. Adenosine Triphosphate Content of Marine Bacteria. Limnol. Oceanogr. 12:319-324.

Holm-Hansen, O. and C.R. Booth. 1966. The Measurement of Adenosine Triphosphate in the Ocean and its Ecological Significance. Limnol. Oceanogr. 11:510.

Holm-Hansen, O. 1969. Determination of Microbial Biomass in Ocean Profiles. Limnol. Oceanogr. 14:740-747.

Holmes, R.W. 1962. The Preparation of Marine Phytoplankton for Microscopic Examination and Enumeration on Molecular Filters. U.S. Fish Wild. Serv. Spec. Sci. Rep. No. 433:1-6.

Instruction Manual 760 Luminescence Biometer. 1960. E.I. DuPont De Nemours and Co., Wilmington, Delaware.

Jackson, H.W. and L.G. Williams. 1962. Calibration and Use of Certain Plankton Counting Equipment. Trans. Amer. Microscop. Soc. 81:96.

Jenkins, D. 1965. Determination of Primary Productivity of Turbid Waters With Carbon-14. J. WPCF. 37:1281-1288.

Jitts, H.R. 1963. The Standardization and Comparison of Measurements of Primary Production by the Carbon-14 Technique. In: Proc. Conf. on Primary Productivity Measurement, Marine and Fresh Water (M.S. Doty, ed.) Univ. of Hawaii, Aug.-Sept. 1961. U.S. Atomic Energy Comm. Div. Tech. Inf. T.I.D. 7633:103-113.

Joint Industry/Government Task Force of Eutrophication. 1969. Provisional Algal Assay Procedure, pp. 16-29.

Kester, E., I. Dredall, D. Connops, and R. Pytowicz. 1967. Preparation of Artificial Sea Water. Limnol. & Oceanogr. 12:176-178.

Laroche, G, R. Eisler, and C.M. Tarzwell. 1970. Bioassay Procedures for Evaluation of Acute Toxicities of Oil and Oil Dispersants to Small Marine Teleosts and Macroinvertebrates. J. Water Pollut. Control Fed. 42:1982-1989.

Lorenzen, C.J. 1966. A Method for the Continuous Measurement of *in vivo* Chlorophyll Concentration. Deep Sea Res. 13:223-227.

Lorenzen, C.J. 1967. Determination of Chlorophyll and Pheopigments: Spectrophotometric Equations. Limnol. & Oceanogr. 12(2):343-346.

Lund, J.W., C. Kipling, and E.D. Lecren. 1958. The Inverted Microscope Method of Estimating Algae Numbers and the Statistical Basis of Estimations by Counting. Hydrobiologia. 11:143-70.

Mackenthun, K.M. 1969. The Practice of Water Pollution Biology. U.S. Dept. of the Interior, FWPCA. 281 pp.

McAllister, C.D. 1961. Decontamination of Filters in the C-14 Method of Measuring Marine Photosynthesis. Limnol. & Oceanogr. 6:477-450.

McNabb, C.D. 1960. Enumeration of Fresh Water Phytoplankton Concentrated on the Membrane Filter. Limnol. & Oceanogr. 5:57-61.

Moss, B. 1967. A Spectrophotometric Method for the Estimation of Percentage Degradation of Chlorophylls to Pheo-pigments in Extracts of Algae. Limnol. & Oceanogr. 12:355-340.

Mullin, M.M., P.R. Sloan, and R.W. Eppley. 1966. Relationship Between Carbon Content, Cell Volume, and Area in Phytoplankton. Limnol. & Oceanogr. 11:307-311.

National Academy of Sciences. 1969. Recommended Procedures for Measuring the Productivity of Plankton Standing Stock and Related Oceanographic Properties. Natl. Acad. Sci., Washington, D.C. 59 pp.

Odum, H.T., W. McConnel, and W. Abbot. 1959. The Chlorophyl "a" of Communities. Pub. Texas Inst. Mar. Sci. 5:65-95.

Palmer, C.M. and T.E. Maloney. 1954. A New Counting Slide for Nannoplankton. Am. Soc. Limnol. Oceanogr. Spec. Publ. No. 21, pp. 1-6.

Schwoerbel, J. 1970. Methods of Hydrobiology (Fresh Water Biology). Pergamon Press, Hungary, pp. 200.

Steeman-Neilson, E. 1952. The Use of Radioactive Carbon (C-14) for Measuring Organic Production in the Sea. J. Cons. Cons. Int. Explor. Mer 18:117-140.

Strehler, B.L. 1968. Bioluminescence Assay: Methods of Biochemical Analysis. (Glictz, D., Ed.) Interscience, New York. Vol. 14, 99 pp.

Strickland, J.D.H. and T.R. Parsons. 1968. A Practical Handbook of Sea Water Analysis. J. Fish. Res. Board Can., Bulletin No. 167, 311 pp.

Talling, J.R. and G.E. Fogg. 1959. Measurements (in situ) on Isolated Samples on Natural Communities, Possible Limitations and Artificial Modifications. In: A Manual of Methods for Measuring Primary Production in Aquatic Environments, R. A. Vollenweider, ed. IBP Handbook, No. 12, F.A. Davis, Philadelphia. pp 73-78.

United Nations Educational, Scientific, and Cultural Organization (UNESCO) 1966. Monographs on Oceanographic Methodology. In: Determination of Photosynthetic Pigments in Sea Water. UNESCO, Paris. 69 pp.

Utermohl, H. 1958. Zur Vervollkommung der Quantitativen Phytoplankton-Methodik. Mit. Int. Ver. Theor. Angew. Limnol. 9:1-38.

Weber, C.I. 1968. The Preservation of Phytoplankton Grab Samples. Trans. Am. Microscop. Soc. 87:70.

Weber, C.I. 1973. Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents. EPA-6704-73-001, U.S. Environ. Prot. Agency Ecol., Cincinnati, OH.

Yentsch, C.S. and J.H. Ryther. 1957. Short-term Variations in Phytoplankton Chlorophyll and Their Significance. Limnol. & Oceanogr. 2:140-142.

Yentsch, C.S. and D.W. Menzel. 1963. A Method for the Determination of Phytoplankton Chlorophyll and Phaeophytin by Fluorescence. Deep-Sea Res. 10:221-231.

B. STATIC METHOD FOR ACUTE TOXICITY TESTS WITH PHYTOPLANKTON

J. H. Gentile and Mimi Johnson

1. Introduction

The method described here is designed for analysis of effects of ocean-dumped material on growth of marine unicellular algae. It involves addition of liquid waste or extracts from sludge to algal growth medium, addition of algae to the medium, and measurement of growth for 96 hours.

Because the capability of calculating EC50 values from bioassay data is required by law, dilutions of ocean-dumped material are necessary. As it is impossible to estimate potential algal toxicity or stimulatory action of each batch of ocean-dumped material, the recommended dilutions may not be sufficient to yield EC50 values in every case. The logistics of algal bioassay are complicated and time-consuming. They must be considered carefully before requirements are imposed.

2. Maintenance of Test Organisms

The marine unicellular algal species to be used is Skeletonema costatum. The algae may be obtained from Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.

The algae are to be maintained in stock culture collections in artificial sea water medium. The artificial sea water is prepared by dissolving artificial sea salts (such as Rila Salts, Rila Products, Teaneck, New Jersey 07666) in glass-distilled water to a salinity of 30 parts per thousand (30 grams of salt in 1000 ml of artificial sea water). Add 15.0 ml of metal mix, 1.0 ml of minor salt mix, and 0.5 ml of vitamin mix to each liter. The compositions of the mixes are given in Table 1-B.

Filter (with suction) the sea water medium through a 0.22μ membrane filter (similar to one manufactured by the Millipore Corporation, Bedford, Massachusetts 01730, Catalog No. GSWP 047 00). Before filtration, pass 1 liter of 0.1 N HCl and 5 liters of glass-distilled water through the filter. Dispense 200 ml of medium into 500-ml Erlenmeyer flasks and use polyurethane foam plugs to seal the flasks. Autoclave at 121°C and 15 lb pressure for 15 minutes. The flasks must be washed with detergent, soaked in 10% HCl, and rinsed 10 times with distilled water.

Equilibrate at room temperature for one day, and check the pH of medium in a flask especially set up for this purpose as above. The pH should be between 7.8 and 8.1. If the pH is not within this range, discard all flasks and make new medium. The pH should fall within this range before a test is started.

Add 10 ml of stock algal culture to each flask and incubate without shaking under 450- to 500-foot candles illumination at $20^{\circ} \pm 2^{\circ}$C with alternating periods of light (16 hours) and darkness (8 hours). Use standard microbiological techniques for flaming the necks of flasks whenever algae are transferred.

Stock cultures as described above must be renewed every 10 days. They need not be shaken during incubation.

3. Preparation of Test Medium

a. Liquid Waste

Liquid waste to be tested must not be modified before use. Liquid samples taken for analysis, however, must be taken in glass containers with Teflon-lined lids. The glassware and liners must be washed with detergent, soaked overnight in 10% HCl, rinsed 10 times with glass-distilled water, rinsed once with acetone, and again rinsed 10 times with glass-distilled water.

Prepare dilutions of liquid waste as follows:

(1) Mix 100 ml of liquid waste with 900 ml of artificial sea water that does not contain trace metal, minor salt, or vitamin mixes. This will be considered to be undiluted medium.

(2) Add 1 part of (1) to 9 parts of artificial sea water. This is a 10% solution of undiluted medium.

(3) Add 1 part of (2) to 9 parts of artificial sea water. This is a 1% solution of undiluted medium.

(4) Add 1 part of (3) to 9 parts of artificial sea water. This is a 0.1% solution of undiluted medium.

b. Sludge

When sludge is tested, artificial sea water without trace metals, minor salts, or vitamins are used as extractant. Salinity of the extractant is 30 parts per thousand and the procedure is:

1. Place a representative portion of the sludge into a 250-ml capacity graduated cylinder, filling to the 250-ml mark. Let the sludge settle overnight (approximately 16 hours). Carefully decant and discard the supernate.

TABLE 1-B. COMPOSITION OF MIXES TO BE ADDED TO ARTIFICIAL SEA WATER

Mix	Amount
Metal mix:[1]	
$Fe Cl_2 . 6 H_2 O$*	0.480 g
$Mn Cl_2 . 4 H_2 O$*	0.144 g
$Zn SO_4 . 7 H_2 O$*	0.045 g
$Cu SO_2 . 5 H_2 O$*	0.157 mg
$Co Cl_2 . 6 H_2 O$*	0.404 mg
H_3BO_3	0.140 g
Distilled water	1ℓ
Vitamin mix:	
Thiamin hydrochloride	50.0 mg
Biotin†	0.01 mg
B_{12}†	0.10 mg
Distilled water	100 ml
Minor salt mix:	
K_3PO_4	3.0 g
$Na NO_3$	50.0 g
$Na_2 SIO_3 . 9 H_2 O$	20.0 g
Distilled water	1ℓ

---

[1] Metal mix should be added after filtration.

*Separate aqueous solutions of these metal salts are maintained at such concentrations that 1 ml of each is added to 1ℓ of mix.

†Biotin is maintained as 1 mg/100 ml alcoholic stock solution; B_{12} in a 10 mg/100 ml aqueous solution.

2. Add 100 ml of the wet settled sludge to a gallon-capacity wide-mouthed jar and add 900 ml of artificial sea water at room temperature. If more growth medium will be required, add more settled sludge and artificial sea water to the jars, but keep the ratio of 100:900 constant. Cap the jars tightly and shake on an automatic shaker at about 100 excursions per minute for 30 min. At the end of the shaking period, remove the jar from the shaker, stand it in an upright position, and let contents settle for 1 hour.

3. Filter the supernatant fluid through glass wool, a membrane filter of 5.0μ porosity, and then through a membrane filter of 0.22μ porosity. When the filters clog, replace them. The filters must be washed before use by passing through them one liter of 0.1 N HCl and 5 liters of glass-distilled water. All glassware associated with filtration must be washed with detergent, soaked overnight in 10% HCl, and rinsed with glass-distilled water before use.

4. The following solutions will be used in the test:

(a) Filtered extract. This will be considered to be undiluted medium.

(b) Add 1 part of (a) to 9 parts of artificial sea water. This is a 10% solution of undiluted medium.

(c) Add 1 part of (b) to 9 parts of artificial sea water. This is a 1% solution of undiluted medium.

(d) Add 1 part of (c) to 9 parts of artificial sea water. This is a 0.1% solution of undiluted medium.

5. After filtration and dilution of liquid or sludge material, add 30.0 ml of metal mix, 2.0 ml of minor salt mix, and 1.0 ml of vitamin mix to each liter and record the pH.

6. Add 48.0 ml of each solution to sterile 125-ml volume Erlenmeyer flasks that were washed with detergent, soaked overnight in 10% HCl, rinsed 10 times with glass-distilled water, rinsed once with acetone, and again rinsed 10 times with glass-distilled water. Prepare three flasks for each solution and for each algal species used. Use polyurethane foam plugs to seal the flasks.

7. Suggested apparatus for extraction, or their equivalent, are:

a. Laboratory shaker, Eberbach 6000 with a 605 Utility Box, or equivalent, capable of shaking a 1-gallon container at 100 excursions per minute.

b. Glass jars, wide mouth, 1-gallon capacity with Teflon lined, screw top lids. Note: If necessary to purchase jars and Teflon sheets separately, the Teflon lid liners can be prepared by the laboratory personnel. Jars and lids should be equivalent in quality to those supplied by the Cincinnati Container Corporation, 2833 Spring Avenue, Cincinnati, Ohio

45225. Jars, Cat. No. 120-400-F-0-0-4 (128 oz.); Lids, Cat. No. 120-400-White, FTK, PPE. Teflon sheets should be equal in quality to those supplied by the Cadillac Plastic Co., 3818 Red Bank Road, Cincinnati, Ohio 45227.

4. Bioassay

a. Preparation of algae

Four days before the bioassay test is performed, add 5 ml of algal stock culture that is at least 5 days old to 45 ml of sterilized artificial sea water that contains trace metals, minor salts, and vitamins as described in Section 2, Maintenance of Test Organisms. Place mixture in 125-ml capacity Erlenmeyer flasks fitted with polyurethane foam plugs. Incubate the new cultures under 450- to 500-foot candles from cool white fluorescent tubes at 20 ± 2°C. Incubate cultures on rotary shaker platforms (No. G2 shaker fitted with No. AG2-125 platform from New Brunswick Scientific Co., New Brunswick, New Jersey 08903, or equivalent) at 140 ± 10 excursions per minute. The lighting cycle should be 16 hours of light followed by 8 hours of darkness.

On the first day of testing, add 1.0 ml of algal culture to a volumetric flask of 25 ml capacity. Bring to approximately half volume with testing medium, add 2 drops of 10% formalin in growth medium, and bring to full volume with testing medium. Wait 5 minutes.

Shake each flask to attain a homogeneous suspension of cells. Remove a sample of the homogeneous suspension quickly with a small pipette and fill each side of a Spencer Bright-Line haemocytometer. Be sure that the suspension does not overflow into the troughs of the haemocytometer. At 100X magnification, count all cells within and impinging upon the 4 1 mm^2 corner squares and the 1 mm^2 central square of each grid. Multiply the count from the 10 squares by 25,000 to find the number of cells in 1 ml of the original suspension.

The object of these counts is to determine the dilution required to attain a final concentration of 100,000 cells per ml in the original cell culture. For example, if the number of cells in a ml of culture is 200,000, then the original culture should be diluted 1:1 with test medium to yield 100,000 cells per ml.

b. Growth of algae

Using sterile pipettes, add 2.0 ml of the algal suspension that contains 100,000 cells per ml to the flasks that were prepared with 48.0 ml of test medium.

Place the flasks on rotary shaker platforms and set the platform at 140 ± 10 excursions per minute. Illuminate with overhead cool flourescent lights. Intensity of light should be between 450- and 500-foot candles with a lighting cycle at 16 hours of light, followed by 8 hours of darkness. The temperature should be $20^{\circ} \pm 2^{\circ}$C.

Incubate the shaking cultures for 96 hours. At that time, add two drops of 10% formalin in artificial sea water to each flask, wait five minutes, swirl the cultures to resuspend the cells to a homogeneous suspension, and count in a haemocytometer as described above.

c. Untreated controls

Control algal cultures must be grown in untreated medium at the time bioassays on liquid waste or sludge are being done. In this case, untreated medium, with its full complement of metal, vitamin, and minor salt mixes, is shaken, filtered, and added to flasks in the same manner as when sludge was extracted. The cell suspension used to inoculate the untreated growth is prepared as described above, except untreated growth medium is used for diluting.

Three flasks are used in growth of controls, and counting is done as described above.

5. Analysis of Results

Calculate the average values for number of algal cells per mililiter in control and each dilution of waste-treated flasks.

EC50 value is the dilution at which waste material causes 50% reduction in growth. In order to estimate this value, inspect the average values to learn if numbers of algal cells in the waste-treated flasks are (1) less than half of those in the untreated control flasks, and (2) more than half of those in the untreated control flasks. To determine an EC50, at least one point must be greater than and one point less than the EC50. Using a semilogarithmic coordinate paper, plot the average cell count for a dilution that yields more than half the average cell count in a dilution that yields less than half of the average cell count of control flasks. The dilution should be plotted on the logarithmic axis and the percentage of growth in relation to the control plotted on the arithmetic axis. Draw a straight line between the two points. The concentration at which this line crosses the 50% growth line is the EC50 value.

C. FLOWING SEA WATER TOXICITY TEST USING OYSTERS (CRASSOSTREA VIRGINICA)

P.A. Butler and J. I. Lowe

The following test procedure is included as a "special bioassay" for evaluating short-term effects of specific wastes on marine mollusks. It is recommended only for use with the commercial Eastern oyster, Crassostrea virginica, and requires flowing unfiltered, natural sea water. This test should be used only with materials which can be dissolved in water or other solvents. The test has proven valuable at ERL, Gulf Breeze, where it has been used for several years to evaluate the effect of insecticides, herbicides, and other toxic organics on oysters (Butler, 1965).

This procedure, described below, is reprinted from a report by the Subcommittee on Mollusks of the Standard Bioassay Committee for the 14th Edition of Standard Methods for the Analysis of Water and Waste Water. It is included in this manual by permission of Dr. Philip A. Butler, Chairman of the subcommittee.

SHELL DEPOSITION TEST

The deposition of new shell in juvenile oysters is directly affected by changes in ambient water quality. The degree of inhibition in shell deposition is quantitatively related to the amount of environmental stress.

This 96-hour test demonstrates the comparative toxicity of pollutants to young oysters. The test is conducted with flowing unfiltered sea water in the temperature range between 15° and 30°C. Actively feeding oysters extend their mantle edges to the periphery of the shell or valves. The body can contract, however, to occupy a much smaller area. If the peripheral valve edges are ground away mechanically, the oysters respond by depositing new shell to replace this loss.

The growth of new shell is primarily linear during the first week, and the rate of deposition is an index of the animal's reaction to ambient water quality. With acceptable water conditions, 25-mm and larger oysters deposit as much as 1.0 mm of peripheral new shell per day. Small oysters (less than 50 mm) are more suitable than large ones because typically they form new shell deposits at temperatures ranging from about 10° to 30°C in contrast to mature oysters, which tend to become less active at temperature extremes.

Test data are independent of minor fluctuations in temperature and salinity during the 96-hour exposure, since the simultaneous shell deposition in control oysters is considered to be the norm or 100 percent.

Procurement and Preparation of Oysters

Oysters, about 25 to 50 mm in height, with reasonably flat, rounded shape, are culled to singles, cleaned, and maintained in trays in the natural environment. At the time of the test, oysters are recleaned and about 3-5 mm of the shell periphery are removed, leaving a smoothly rounded blunt profile. This is conveniently done by hand-holding the oysters against an electric disc grinder. Removal of too wide a rim of shell will make an opening into the shell cavity; damaged oysters should be discarded.

Test aquaria can be fabricated of glass or fiberglassed wood, and should measure about 64 x 38 x 10 cm deep (25 x 15 x 4 inches) to provide adequate space for 20 oysters. Such containers permit adequate circulation of the water, while avoiding physical agitation of the oysters by the water current.

The unfiltered water supply in a constant head reservoir is delivered by calibrated siphons to the aquaria via a mixing trough into which the toxicant is also metered in an appropriate solvent. Stock solutions of the toxicant are prepared so that a delivery of 1 or 2 ml per minute by means of a calibrated pump will result in the desired concentration. Baffles in the trough ensure adequate mixing and aeration before the water enters the test aquaria.

The aquaria contain about 18ℓ at 75 percent capacity and with a flow rate of 100ℓ $hour^{-1}$ will provide 5ℓ of water $hour^{-1}$ $oyster^{-1}$. Small oysters feed and grow readily under these conditions.

Bioassay Procedure

Oysters are prepared and randomly distributed so that each control and test aquarium contains 20 individuals. Oysters are placed with the left, cupped-valve down; the anterior hinged ends are oriented in one direction. One control aquarium is established to receive only the toxicant solvent; one aquarium is established for each desired concentration of the toxicant.

At the end of 96 hours, all oysters are removed from the water and the shell increments are measured. Shell deposition is not uniform on the periphery, therefore the length of the longest "finger" of new shell on each oyster, measured to the nearest 0.5 mm, is recorded.

Calculation

The ratio of the mean growth of a group of test oysters to the mean growth of the control oysters provides a percentage index of the tolerance of the oysters to a specified toxicant concentration. A 96-hour EC50 (concentration inhibiting shell deposition by 50%) may be calculated from an appropriate exposure series for the indicated test conditions. These values are relative and may differ significantly under different salinity or temperature regimes. Appropriate statistical techniques should be used to determine confidence limits when possible.

A preliminary exposure series is helpful in establishing a suitable range of toxicant concentrations. In general, three or four oysters exposed for 48 hours to appropriate concentrations of the test material will bracket the range of toxicant concentrations required to determine 96-hour EC50 data.

REFERENCE

Butler, Philip A. 1965. Reaction of Some Estuarine Mollusks to Environmental Factors. In: Biological Problems In Water Pollution - Third Seminar - 1962. U.S. Department of Health, Education, and Welfare, Public Health Service Publication No. 999-WP-25 June, 1965.

D. METHODS FOR THE CULTURE AND SHORT TERM BIOASSAY OF THE CALANOID COPEPOD (ACARTIA TONSA)

John H. Gentile and Suzanne Lussier Sosnowski, Environmental Research Laboratory, Narragansett, RI

INTRODUCTION

The methodology described in this section is designed to provide bioassay data on the effects of a toxicant on a marine copepod. The techniques described have been used for several years by EPA and represent the synthesis of many researchers' efforts from both government and universities. Basically, dose response curves are constructed from mortality data collected from 24-, 48-, 72-, and 96-hour exposure observations. While these observation intervals should be considered a basic requirement, more frequent observations or longer exposures may be necessary. From the above observations, estimates of the LC50 and 95 percent confidence limits can be determined (Litchfield and Wilcoxon, 1949; Finney, 1964, 1971; Standard Methods, 1971, 13th Edition).

COLLECTION AND PREPARATION OF SEA WATER

The sea water for both culture and bioassay, if possible, should be collected from the study area. First, the sea water, when adjusted to 30 °/oo salinity and 20°C, must support survival of the adult copepod *Acartia tonsa* for the 96-hour bioassay period. A second and more demanding requirement is that, with the proper enrichments, sea water supports growth of the food algae and the complete life cycle of the test species. If no suitable natural sea water is available, a synthetic sea water formulation may be employed (Appendix 1-D).

Niskin or Van Dorn samplers can be used to collect sea water from three to ten meters depth to avoid surface contamination. Collected sea water can be transported to the laboratory in glass or polyethylene carboys that have been aged with sea water. In the laboratory, the water is filtered through a 1.0 acid washed filter (glass fiber, cellulose, acetate, nylon or polycarbonate) to remove particulate matter and stored at 4°C in the above containers.

Measurements of salinity, dissolved oxygen, and pH should be recorded at the time of collection.

ALGAL FOOD CULTURES

Although a variety of algal diets have been used for copepod cultures (Zillioux and Wilson, 1966; Heinle, 1969; Katona, 1970; Nassogne, 1970), the following modification of Wilson and Parish (1971) has been used successfully in the EPA Environmental Research Laboratory in Narragansett (Table 1-D). We have added Skeletonema costatum because it is a naturally occurring food for Acartia tonsa.

TABLE 1-D. COMPOSITION OF ALGAL DIET AND RECOMMENDED CONCENTRATION FOR FEEDING, EGG LAYING, AND NAUPLIAR FEEDING

Item	Adult & Copepodite	Naupliar	Egg Laying
Skeletonema costatum	5.0×10^6	5.0×10^5	1.5×10^7
Thalassiosira psuedonana	7.0×10^6	7.0×10^5	2.1×10^7
Isochrysis galbana	5.0×10^6	5.0×10^5	1.5×10^7
Rhodomonas baltica	3.0×10^6	3.0×10^5	9.0×10^6
Total cells/liter	2.0×10^7	2.0×10^6	6.0×10^7

These algae are grown axenically in filtered natural or synthetic sea water at 30 ^{o}/oo salinity and 20°C with 2500-5000 lux continuous illumination or 14L:10D. The nutrient enrichments are modifications of those of Guillard and Ryther (1962)(Appendix 2-D).

Algal cultures may be grown either in standard test tubes or flash cultures if desired; or in the fill and draw semi-continuous system described below.

Enriched sea water is dispensed into either screw-capped test tubes (50 ml) or Erlenmeyer flasks fitted with Teflon lined caps. After autoclaving (15 min @ 15 psi & 250°F), the medium is allowed to cool and equilibrate with atmospheric gases for 48 hours. Sterility checks are made on each set of autoclaved medium by randomly selecting a representative number of tubes or flasks and inoculating one ml of their contents into tubes of sterility check medium (Appendix 2-D). Caps are tightened and the inoculated tubes stored in darkness for up to two weeks. The appearance of turbulence or opalescence in the test medium indicates the presence of contamination.

Tubes or flasks are inoculated with each alga on a regular basis to continually provide a log-phase, high density food source, the frequency being determined from interpretation of algal growth curves. The cultures should be harvested at their maximum log-growth phase cell density. Although this system works, it is very time consuming since it requires frequent cell counts and a large turnover of glassware.

The recommended algal culture system is of a fill-draw type in which cultures are easily maintained near their maximum log-phase cell density and growth rate (Appendix 3-D). It is then a simple matter to draw off a constant volume and replace it with fresh medium so that within 24 hours the culture will have reached the same cell density. When intervals longer than 24 hours occur between harvests, proportionally greater amounts of culture are drawn off and replaced. This system can be scaled up or down, depending on food needs. But most importantly, this system produces algal food that is physiologically and nutritionally consistent. Thus the nutritional history of the test species is better controlled. If this system is used, a series of tube cultures of each of the four algal foods must be maintained concurrently in case of contamination of the large cultures.

Algal cell densities may be determined in a variety of ways. Direct microscopic counts can be made with a haemocytomer, Palmer-Maloney chamber, or Utermohl chamber (inverted scope) (Schwoerbel, 1970) (Standard Methods, 1971). In addition, an electronic particle counter is an accurate and rapid method for determining unialgal densities. Finally, manual counts, if necessary, can be related to chlorophyl absorbance at 440 mμ or 665 mμ, using a spectrometer. A curve that compares cells/ml with absorbancy should be prepared from serial dilutions of each algal culture. Then a rapid and simple measure of absorbancy can be used to replace the cell count.

ZOOPLANKTON CULTURE

Collection

Zooplankton (including Acartia tonsa) are collected by slowly ($\leq$4 km/hr) towing a plankton net (aperture 150 to 250 μm at a depth of one to three meters). Captured animals are carefully transferred to insulated containers three-fourths filled with ambient sea water. The population density should not exceed ca. 25/ℓ to assure that the dissolved oxygen concentration remains adequate if the organisms are not returned to the laboratory within one to two hours. It is imperative to measure and record the temperature and salinity at the time of the collection since these parameters must be maintained during the initial stages of laboratory culture.

Holding

In the laboratory, the samples immediately are transferred to 2.3ℓ (190 x 100mm) borosilicate crystallizing dishes. Volume is adjusted to 2000 ml with filtered sea water at ambient temperature and salinity; each dish is then fed the adult algal diet (Table 1-D). The cultures are incubated at ambient temperature and 14L:10D cool white illumination of 1000 lux. After 24 hours, acclimation of the cultures to 20°C and 30 ^{o}/oo salinity should commence. Salinity and temperature increments of 5 ^{o}/oo and 5°C per day are satisfactory. Organisms can remain in the original vessel and culture volumes can be changed by alternately siphoning through 150-μm plankton netting and adding sea water of a different salinity. Transfers are made by carefully pipetting or slowly siphoning organisms to new vessels. During acclimation, a daily feeding schedule is maintained.

Holding and acclimation can also be accomplished by adding the tow collections to 4-12ℓ aspirator bottles equipped with low rpm ($\leq$ 25 rpm) motors.* Organism density should be adjusted to 1:10 ml of culture volume.

Sorting and Identification

The plankton tow contains a mixture of species from which *Acartia tonsa* must be isolated. For basic information on the taxonomy and biology of the genus *Acartia* and other coastal calanoids, the following papers are recommended (Conover, 1956; Heinle, 1966, 1969; Wilson, 1932; Rose, 1933; Fraser and Hansen, Eds., *Serie Fiches Identification Zooplancton*). (See Appendix 4-D for comparison of calanoid copepods usually occurring with *Acartia tonsa*.)

To facilitate capture of organisms, the culture volume is reduced from 2000 ml to 500 ml by slowly siphoning sea water, using 150-μm plankton netting over the siphon intake. Individual adult organisms are attracted to the edge of the dish with a dim light (440-1400 lux) and carefully drawn up into a wide-bore ($\geq$ 2mm) transfer pipette. Individual animals are placed in depression slides and identified microscopically. Once the investigator becomes familiar with *Acartia* morphology and swimming behavior (short spurts as opposed to long glides), it will not be necessary to identify animals by fifth leg. With practice, an investigator can examine several animals simultaneously under low magnification. Extraneous species then are removed and *Acartia* are transferred to food-enriched filtered sea water at 30 ^{o}/oo and 20°C. Contamination of species is prevented by excluding all naupliar and juvenile forms.

Mass Culture

The objective of this system is to provide large quantities of *Acartia tonsa* of standard age for short-term bioassays.

The mass culture unit is derived from culture systems used by Mullin and Brooks (1967) and Frost (1972). The culture vessel is a pyrex aspirator bottle whose size can range from 4.0 to 40 liters depending on the number of copepods needed. The contents are gently mixed by a low rpm motor* ($\leq$25 rpm) mounted above the culture vessel. Thus, algal food is suspended where these planktonic copepods normally feed. It must be emphasized that water movement is gentle and free of vortices such as produced by magnetic stirrers (Figure 1-D). Cool white fluorescent lights provide 2000 lux illumination incident to the culture surface on a 14L:10D cycle.

Acartia tonsa females are capable of producing more than 30 eggs per female per day when fed the adult algal food ration recommended in Table 1-D (Wilson and Parrish, 1971). Thus, if 250 or more gravid females are brooded, theoretically, more than 5,000 eggs will be produced within 24 hours. For this potential number of adults, a 40-liter culture vessel would be desirable. Generally, the relationship between culture volume (mls) and organism density is 10 ml:1.

---

*W.W. Grainger, Inc. Dayton Shaded Pole Gearmotors, 20 rpm. All angle Operation #2Z808.

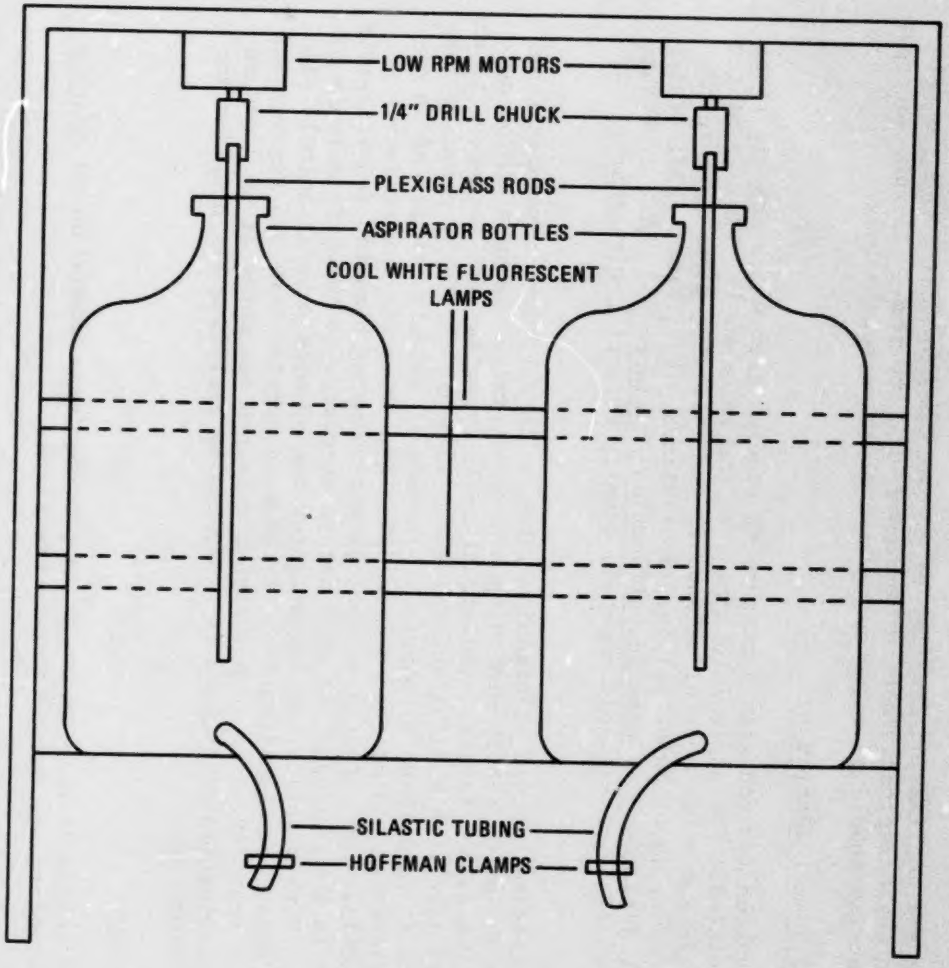


Figure 1-D. Mass Copepod Culture Systems (Static).

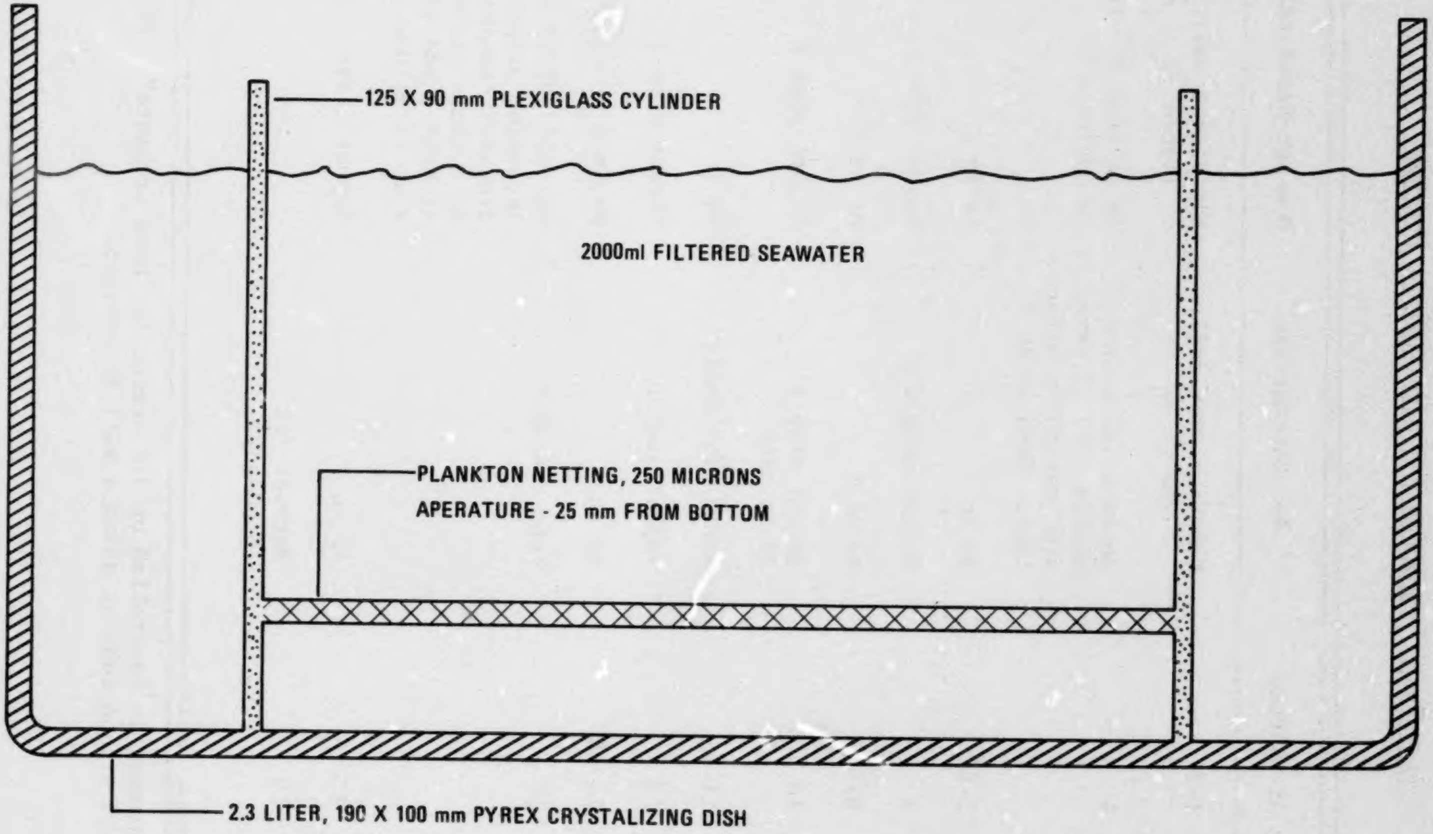

125 X 90 mm PLEXIGLASS CYLINDER
2000ml FILTERED SEAWATER
PLANKTON NETTING, 250 MICRONS
APERATURE - 25 mm FROM BOTTOM
2.3 LITER, 190 X 100 mm PYREX CRYSTALIZING DISH

Figure 2-D. Generation Cage (after Heinle) (personal communication).

TABLE 2-D. PROTOCOL FOR MASS COPEPOD CULTURE

Step	Day of Culture	Age-Standardized	Non-Age-Standardized
1	1-3	Naupliar diet daily (Table 3)	Adult diet daily (Table 3)
2	4	Replace 50% culture medium with filtered s.w. retaining organisms. Feed as in 1	As in step 2. Feed as adults.
3	5-6	As in 1	As in 1
4	7	Repeat step 2	Repeat step 2
5	8-9	As in 1	As in 1
6	10	Repeat step 2 Adult diet	Repeat step 2
7	11-12	Adult diet daily	As in 1
8	13	Repeat step 6	Repeat step 2
9	14-15	As in 7	As in 1
10	16	Repeat step 6	Harvest 33% of culture including organisms. Transfer remaining 67% to a clean carboy by siphon* & add filtered s.w. to volume.
11	17-18	As in 7	Repeat steps 1-10
12	19	Harvest for Bioassays	---

*Rate of siphoning is controlled by difference in "head pressure". Do not constrict the siphon tube or animals will be damaged.

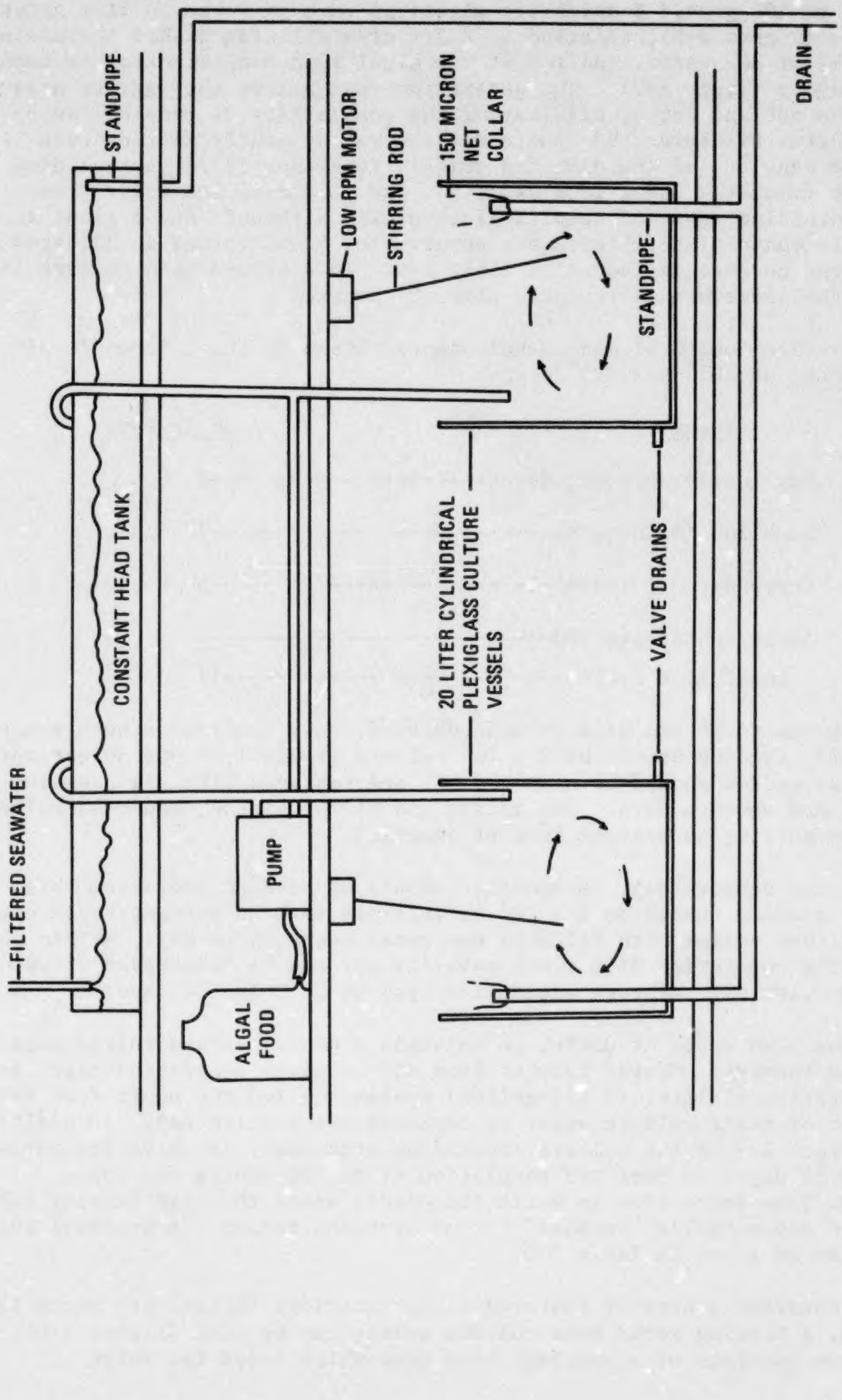


Figure 3-D. Mass Copepod Culture (Flowing).

Fifty to 100 gravid females are placed in each of three to five generation cages (Figure 2-D), immersed in 2.3/ℓ crystallizing dishes containing ca. 2000 ml of sea water, and fed at the algal food concentration recommended for egg-laying (Table 1-D). The generation cage allows the eggs to pass through the net and hatch, eliminating the possibility of cannibalism by adults. After 24 hours, the adults are removed by gently lifting each generation cage out of the dish and quickly immersing it in another dish with three times the usual food density. The remaining sea water from all dishes containing eggs and nauplii is carefully siphoned into a glass aspirator bottle containing filtered sea water. The final volume is adjusted and the naupliar culture is fed as in Table 1-D. If a second mass culture is desired, the procedure is repeated after 24 hours.

The average length of each developmental stage in the life cycle of Acartia tonsa at 20°C and 30 ^{o}/oo is:

Stage	Length in Days
Egg (newly oviposited)	1
Nauplius (6 instars)	7
Copepodite (6 instars)	6
Adult (until gravid)	3
Total Life Cycle	17

During the first six days of mass culture, only naupliar stages are present. Daily feeding should be 2 x 10^6 cells/ℓ (Table 1-D) and 50 percent of the culture medium should be siphoned off and replaced with clean medium on the third and seventh days. The intake end of the siphon should be covered with 60 μm netting to prevent loss of nauplii.

After the seventh day, copepodites should be present and, from this point on, feeding should be 2 x 10^7 cells/ℓ/day with 50 percent replacement of the culture volume with filtered sea water every third day. Within 16 to 17 days, the population will reach maturity and can be bioassayed or used to start new cultures. Average adult life span at 20°C is $\leq$30 days.

We have also found it useful to maintain a non-age-standardized mass culture in reserve. Gravid females from the original generation cages are used to start a 12-liter (3 1/2-gallon) system and fed the adult food ration; 50 percent of their culture water is replaced every third day. In addition, approximately 1/3 of the culture (including organisms) is harvested periodically (10-14 days) to keep the population at ca. 50 adults and copepodites/ℓ. This precaution is worth the effort since the high density cultures have occasionally "crashed" for no apparent reason. A protocol for this system is given in Table 2-D.

If a constant source of filtered (1.0μ cartridge filter) sea water is available, a flowing water mass culture system can be used (Figure 3-D). This system consists of a constant head tank which feeds two large cylindrical reaction vessels. Dilution water flow is controlled by capillary restriction or clamps. The four species algal food is proportionally metered by peristatic pump to provide a constant cell density of 25×10^7 cells/ℓ. This cell density can sustain culture densities in excess of 100 adults and copepodites/ℓ, though harvesting is recommended to keep the density at 50/ℓ.

The reaction vessels are 30 cm high, 30 cm in diameter, and have a 25 cm standpipe. This provides approximately 18ℓ culture volume. The stand pipe has a collar of 150μm nitex net which effectively retains both eggs and nauplii even though they are considerably smaller than the pores. Too fine a net produces excessive clogging. It is likely that bacterial and algal growths reduce the effective mesh size to occlude particles as small as 50 μm. This net collar requires periodic brushing to maintain effective drainage. The reaction vessels, illuminated as in the static system, are equipped with low rpm ($\leq$25) motors to maintain the population in suspension. The dilution rate, approximately 10 ml/min, effectively replaces 50 percent of the culture volume every 24 hours, although the total volume pumped is 80 percent of the reaction volume. Flow rates > 10 ml/min can be used, with caution, to avoid washing out eggs, nauplii, or both.

Harvesting

Mass cultures of copepods that have reached the adult stage are harvested for bioassays as follows: the culture volume is reduced by 75 percent, using a slow siphon whose intake is covered with 60 μm plankton netting. The remaining 25 percent of the culture, including organisms, is carefully transferred to 2.3ℓ pyrex crystallizing dishes (ca. 2000 ml/dish). This transfer is critical and is best performed as follows: because of fragility of the organism, do not constrict the discharge tube to reduce flow. Discharge flow through the ventral tubulation on the aspirator is controlled by minimizing the head pressure between the culture vessel and the crystallizing dish. A slow flow minimizes turbulence and opportunity for organisms to collide into vessel walls.

Harvested animals can be concentrated in the crystallizing dishes by further siphoning the culture medium. Capture is facilitated by using positive phototactive response of the animals.

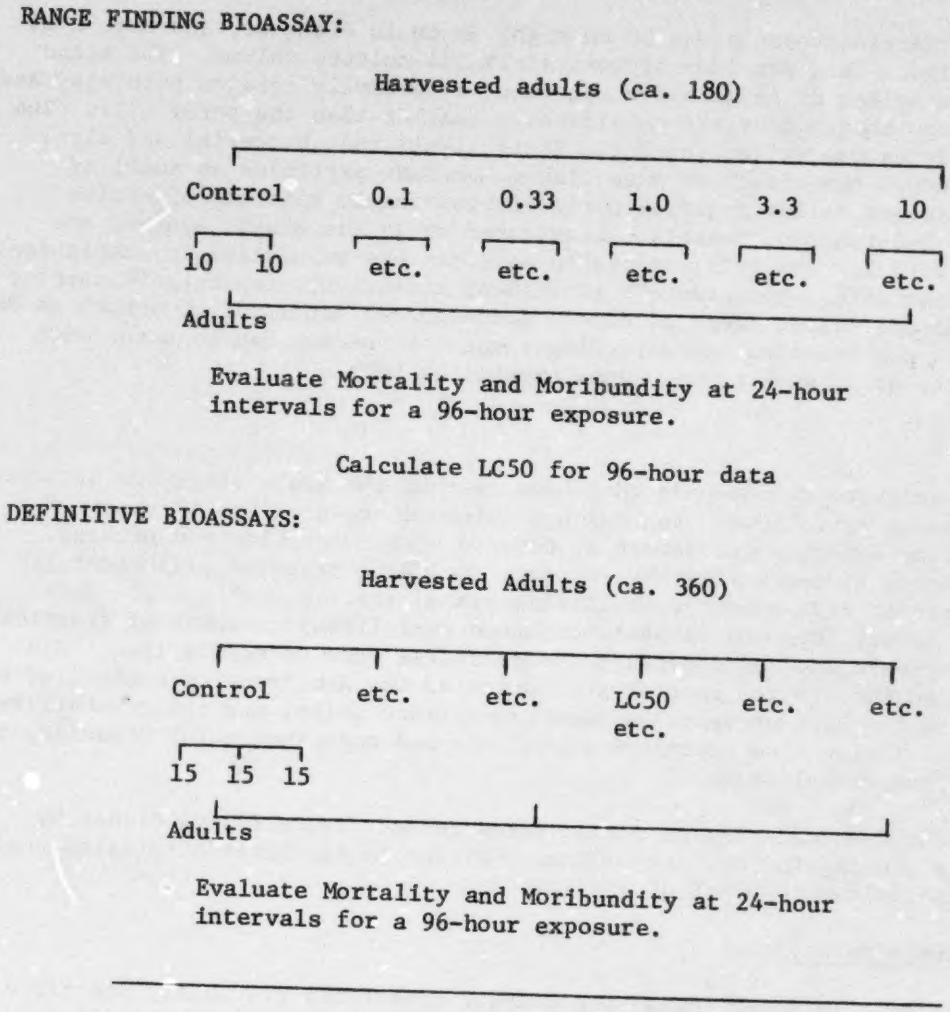
Short-Term Bioassays

Adult *Acartia tonsa* (Dana) and culture conditions previously described are required for the following short-term bioassays. (See Figure 4-D.)

Range-Finding Bioassays

1. Ten adult *Acartia* are tested per replicate with two replicates required per test concentration and control. Feeding is omitted for the duration of the assay. A solvent control must be included when appropriate.

2. Test container must be a suitable flatbottom borosilicate glass dish containing 100 ml sea water. The depth of medium must be $\geq$2.0 cm.



Evaluate Mortality and Moribundity at 24-hour intervals for a 96-hour exposure.

Calculate LC50 for 96-hour data

Evaluate Mortality and Moribundity at 24-hour intervals for a 96-hour exposure.

Figure 4-D. Bioassay protocol.

3. Toxicant concentration selection

Generally, a broad range of concentrations covering at least three orders of magnitude is chosen initially. This is followed by a progressive bisection of intervals on a logarithmic scale (see Standard Methods, 1971).

4. Toxicant administration

a. Water miscible toxicants are added immediately prior to the addition of the test species.

b. Water immiscible toxicants are dissolved in a suitable solvent prior to addition to the test medium. Solvent evaluation must be performed to insure solvent concentrations used are not toxic.

5. Ten adult Acartia are captured from stock cultures with a wide-bore transfer pipette and transferred to a 20-ml beaker containing undosed filtered sea water (ca. 5 ml). Adjust the final volume of this beaker to 15 ml. The animals and the 15 ml of medium are added to 85 ml of toxicant-dosed medium by immersing the beaker and gently rinsing.

6. Exposure period is 96 hours. The number of dead and moribund copepods are observed and recorded at 24, 48, 72, and 96 hours of exposure. To ascertain death, gently touch a motionless animal with a sealed glass capillary probe. Dead animals are removed at each observation point. Control mortalities in excess of 15 percent invalidate the experiment.

7. At each observation period, dissolved oxygen and pH should be measured, particularly if wastes contain large amounts of organic matter. Since the test species is very sensitive to agitation, these measurements at all test concentrations must be made on a series of concurrently prepared unioculated samples.

Definitive Short-Term Bioassay

General culture conditions and handling follow previous discussions. The specifications for this assay are:

(1) Fifteen adults are to be tested in each of three replicates per toxicant concentration and control.

(2) Test vessels are described above.

(3) Concentration ranges for toxicant must include at least two levels above and below the 96-hour LC50 determined from range finding bioassays.

(4) Exposure and data collection are described above.

(5) Calculations and data presentation are as described in Standard Methods (14th Ed.) pp. 565-577. Alternate methods of data presentation are desirable, particularly the application of confidence limits. (See Litchfield and Wilcoxon, 1949, and Finney, 1964, 1971.)

COMMENTS

The bioassay methodology is, at best, a general framework that is subject to modifications as determined by the type of toxicant and the experimental design. For example, in assays with toxicants that readily adsorb to container walls and fail to remain in solution, transfer of organisms to freshly dosed media is required. The frequency of transfer is determined after rates of solubility and adsorption are known.

The mass culture system described can be used as a holding and acclimation system for indigenous populations. For example, in many geographical areas, A. tonsa is replaced by A. clausi during the winter months. Using the above system, we have held A. clausi at 10°C for several weeks. These organisms were used in bioassays at 10°C with excellent results. Thus, we feel that this system, with appropriate modifications, can be used to hold and culture a variety of zooplankters.

In the event that natural sea water is not suitable for survival, growth, and reproduction of the test species, the following synthetic formulations are recommended. The formulation in Appendix 1-D has been used for both whole life history culture and numerous bioassay studies at this laboratory. Heinle (1969) found the commercial sea water Instant Ocean, suitable for the culture of both A. tonsa and E. affinis. Data are not available on the use of Instant Ocean in bioassays or regarding a comparison to natural sea water. Therefore, Instant Ocean is recommended only for culture, not for bioassays, until suitable comparative data are available.

REFERENCES

American Public Health Service. 1971. Standard Method for the Examination of Water and Wastewater. 13th ed. New York. 874 p.

Conover, R.J. 1956. Oceanography of Long Island Sound, 1952-1954. VI. Biology of *Acartia clausi* and *A. tonsa*. Bull. Bingham Oceanogr. Collect. Yale Univ. 15:156-233.

Davey, E.W., J.H. Gentile, S.J. Erickson, and P. Betzer. 1970. Removal of Trace Metals from Marine Culture Medium. Limnol. & Oceanogr. 15:486-488.

Finney, D.J. 1964. Statistical Method in Biological Assay. 2nd ed. Hafner Publishing Co., New York. 668 p.

_____. 1971. Probit Analysis. 3rd ed. Cambridge Univ. Press. London. 333 p.

Fraser, J.H., and V. Kr. Hansen, eds. Serie Fiches Identification Zooplancton.

Frost, B.W. 1972. Effects of Size and Concentration of Food Particles on the Feeding Behavior of the Marine Planktonic Copepod *Calanus pacificus*. Limnol. & Oceanogr. 17(6):805-815.

Gentile, J.H., J. Cardin, M. Johnson, S. Sosnowski. 1974. Power Plants, Chlorine, and Estuaries. Amer. Fish. Soc., 36th Annu. Meeting, Honolulu, Sept. 9-11.

Guillard, R.R., and J.H. Ryther. 1962. Studies of Marine Planktonic Diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacia* (Cleve) Grant. Can. J. Microbiol. 8:299-339.

Heinle, D.R. 1966. Production of a Calanoid Copepod, *Acartia tonsa*, in the Patuxent River Estuary. Chesapeake Sci. 7:59-74.

_____. 1969a. Effects of Temperature on the Population Dynamics of Estuarine Copepods. Ph.D. Thesis, Univ. Maryland, College Park. 132 p.

_____. 1969b. Culture of Calanoid Copepods in Synthetic Sea Water. J. Fish. Res. Bd. Can. 26(1):150-153.

Katona, S.K. 1970. Growth Characteristics of the Copepods *Eurytemora affinis* and *E. herdmani* in Laboratory Cultures. Helgoländer wiss. Meeresunters. 20:373-384.

Kester, E., I. Dredall, D. Connors, and R. Pytowicz. 1967. Preparation of Artificial Sea Water. Limnol. & Oceanogr. 12(1):176-178.

Litchfield, J.T., and F. Wilcoxon. 1949. A Simplified Method of Evaluation Dose-Effect Experiments. J. Pharmacol. Exper. Ther. 96(2):99-115.

Mullin, M.M., and E.R. Brooks. 1967. Laboratory Culture, Growth Rate, and Feeding Behavior of a Planktonic Marine Copepod. Limnol. & Oceanogr. 12:657-666.

Nassogne, A. 1970. Influence of Food Organisms on the Development and Culture of Pelagic Copepods. Helgoländer wiss. Meeresunters. 20:333-345.

Rose, M. 1933. Faune de France. No. 26. Copepodes Pélagiques. Librairie de la Faculté des Sciences. Reprinted 1970 by Kraus Reprint, Nendeln Leichtenstein.

Schwoerbel, J. 1970. Methods of Hydrobiology. Pergamon Press, New York.

Wilson, C.B. 1932. The Copepods of the Woods Hole Region, Massachusetts. Smithsonian Institute, U.S. National Museum Bulletin 158.

Wilson, D.F., and K.K. Parrish. 1971. Remating in a Planktonic Marine Calanoid Copepod. Mar. Biol. 9:202-204.

Zillioux, E.J., and D.F. Wilson. 1966. Culture of a Planktonic Calanoid Copepod through Multiple Generations. Science 151:996-998.

APPENDIX 1-D. SYNTHETIC SEA WATER FORMULATION*

Chemical	g/ℓ
NaCl	24.00
Na_2SO_4	4.00
$CaCl_2.2H_2O$	1.47
$MgCl_2.6H_2O$	10.78
KCl	0.70
H_3BO_3	0.03
$NaHOO_3$	0.20

*Medium is modified from Kester et al. (1967). Salinity is 34 ^{o}/oo and pH 8.0 and must be adjusted to 30 ^{o}/oo with distilled or deionized water. Trace metal contaminants from major salts must be eliminated by ion exchange stripping (Davey et al., 1970). Na_2EDTA (300 mgs/ℓ) may be used for holding and culture, but must be omitted in bioassay studies with trace metals.

APPENDIX 2-D. SEA WATER AND STERILITY ENRICHMENT

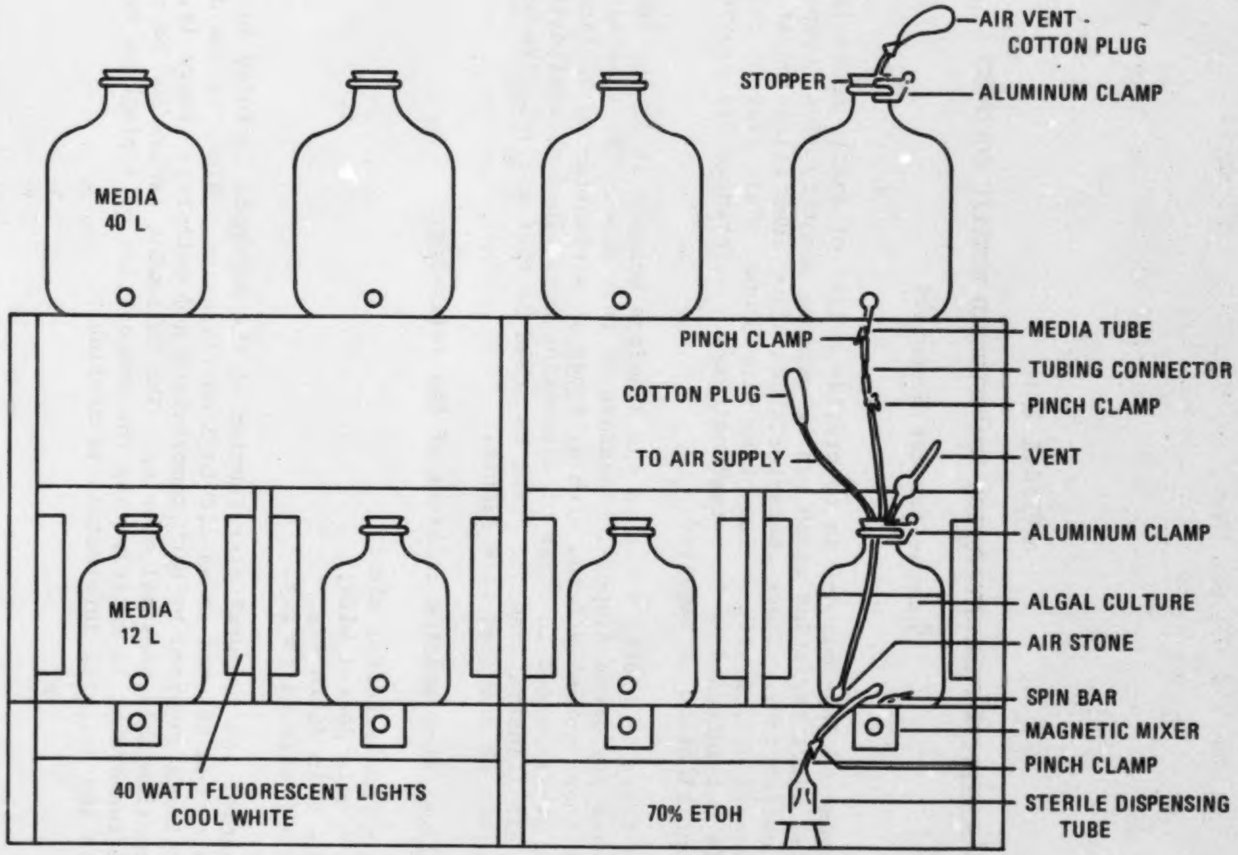
(A) Sea water enrichments for stock algal culture maintenance (After Guillard and Ryther, 1962):

Item	Amount
$NaNO_3$	75 mg/liter
$NaH_2PO_4.H_2O$	5 mg/ℓ
$Na_2SiO_3.9H_2O$	10 mg/ℓ
Vitamins:	
Thiamine HCl	0.10 mg/ℓ
Biotin	0.50 μg/ℓ
B_{12}	0.50 μg/ℓ
Trace Metals:	
$CuSO_4.5H_2O$	0.002 mg/ℓ
$ZnSO_4.5H_2O$	0.004 mg/ℓ
$CoCl_2.6H_2O$	0.002 mg/ℓ
$MnCl_2.4H_2O$	0.036 mg/ℓ
$NaMoO_4.2H_2O$	0.001 mg/ℓ
Fe-sequestrine	1.0 mg (0.13 mg Fe)/ℓ

Buffer:
TRIS-500 mg/ℓ @ pH 7.8-8.2

(B) Sterility enrichments to be added to enriched sea water medium above before autoclaving:

Sodium Glutamate	250 mg/ℓ
Sodium Acetate	250 mg/ℓ
Gycline	250 mg/ℓ
Nutrient Agar	50 mg/ℓ
Sucrose	250 mg/ℓ
Sodium Lactate	250 mg/ℓ
L & D Alanine	250 mg/ℓ



APPENDIX 3-D. Algal culture.

APPENDIX 4-D

DESCRIPTIVE CHARACTERISTICS FOR SELECTED NERITIC COPEPODS

Suzanne Lussier Sosnowski

The purpose of Appendix 4-D is to provide a list of easily recognizable morphological characteristics which can be used to identify live copepods from field collections. These characteristics allow identification at less than 100x magnification with a dissecting microscope. This list of characteristics is not intended to be a taxonomic key. References are provided if further identification is required.

Acartia tonsa is placed first in the drawings because it is the recommended species for ocean disposal bioassays at this time. The remaining species are those normally found with *A. tonsa* in a plankton collection. The species are arranged in order of decreasing morphological similarity to *A. tonsa*. All illustrations are drawn to scale so that the relative size of the species may be compared at a glance.

Each illustrated species consists of the following:

A - female dorsal view;
B - male dorsal view;
C - male fifth leg;
D - female fifth leg.

The spatial and temporal distribution of the copepods included in this appendix also can be used as an aid to identification. Distribution of these species is governed by both temperature and salinity. There is, however, considerable seasonal overlap. The following tables can be used to assist the researcher in anticipating the composition of a plankton tow when temperature and salinity information is available.

APPENDIX 4-D (Continued)

DESCRIPTIVE CHARACTERISTICS FOR SELECTED NERITIC COPEPODS

Salinity

Euryhaline
(10-35 ^{o}/oo)

Acartia tonsa
Acartia clausi
Acartia longiremis
Eurytemora affinis
Eurytemora americana
Eurytemora herdmani
Pseudodiaptomus coronatus
Temora longicornis

Stenohaline
(25-35 ^{o}/oo)

Centropages typicus
Centropages hamatus
Tortanus discaudatus

Temperature

Not > 20° C

Acartia clausi
Pseudocalanus minutus elongatus
Eurytemora herdmani
Tortanus discaudatus

Not < 10° C

Acartia tonsa
Eurytemora affinis
Pseudodiaptomus coronatus
Centropages typicus
Oithona similis

Species	Descriptive Characteristics
Acartia tonsa ♂ 1.00-1.15 mm ♀ 1.25-1.50 mm	1. Spindle-shaped body 2. Urosome 1/3 length of metasome 3. Caudal rami as long as wide 4. Long hairs on first antennae 5. First antennae nearly straight on female, but with acute bend near proximal end on male 6. No egg sacs present 7. Swims in short spurts

Continued

APPENDIX 4-D (Continued)

DESCRIPTIVE CHARACTERISTICS OF SELECTED NERITIC COPEPODS

Species	Descriptive Characteristics
Acartia clausi ♂ 1.00-1.10 mm ♀ 1.15-1.25 mm	1. Spindle-shaped body 2. Urosome 1/3 length of metasome 3. Caudal rami twice as long as wide 4. Long hairs on first antennae 5. First antennae nearly straight on female, but with acute bend near proximal end on the male 6. Three or four pairs of blue dots on ventral surface of metasome, visible only on fresh tow material; preservative causes pigment to fade 7. No egg sacs present 8. Swims in short spurts
Acartia longiremis ♂ 0.8-1.0 mm ♀ 0.9-1.1 mm	1. Spindle-shaped body 2. Urosome 1/3 length of metasome 3. Caudal rami 2-3 times longer than wide 4. Long hairs on first antennae 5. First antennae of male have small hinge 6. Fifth segment with delicate spine on dorsal surface of each posterior corner 7. No egg sacs 8. Swims in short spurts
Pseudocalanus minutus elongatus ♂ 1.00-1.25 mm ♀ 1.20-1.60 mm	1. Body shape elliptical 2. Urosome 1/2 as long as metasome 3. Caudal rami longer than anal segment 4. Short hairs on first antennae 5. Animals from fresh tow have reddish color 6. Female lacks fifth pair of legs 7. Female often with single egg sac 8. Swims with long gliding motion

Continued

APPENDIX 4-D (Continued)

DESCRIPTIVE CHARACTERISTICS OF SELECTED NERITIC COPEPODS

Species	Descriptive Characteristics
Eurytemora affinis ♂ 1.4-1.6 mm ♀ 1.4-1.5 mm	1. Body bullet-shaped 2. Female with large triangular "fenders" at posterior corners of fifth segment 3. Very long caudal rami 4. Spines cover the anal segment and caudal rami 5. Short hairs on first antennae 6. Right first antenna of male is hinged 7. Female often with single large egg sac 8. Swims with gliding motion
Eurytemora americana ♂ 0.75-0.95 mm ♀ 1.60-1.85 mm	1. Body bullet-shaped 2. Female with large "fenders" with sharp spines at posterior corners of fifth segment 3. Very long caudal rami 4. Spines cover the anal segment and caudal rami 5. Short hairs on first antennae 6. Right first antenna thickened and hinged 7. Female often with single large egg sac 8. Swims with gliding motion
Eurytemora herdmani ♂ 1.2-1.5 mm ♀ 1.3-1.6 mm	1. Body bullet-shaped 2. Female with large triangular "fenders" at posterior corners of fifth segment reaching beyond genital segment 3. Very long caudal rami 4. Short hairs on first antennae 5. Right first antenna of male hinged 6. Female often with single large egg sac 7. Swims with gliding motion

Continued

APPENDIX 4-D (Continued)

DESCRIPTIVE CHARACTERISTICS OF SELECTED NERITIC COPEPODS

Species	Descriptive Characteristics
Pseudodiaptomus coronatus ♂ 1.00-1.25 mm ♀ 1.25-1.50 mm	1. Body bullet-shaped 2. Female genital segment swollen with patches of bristles and spines protruding ventrally 3. Caudal rami 2-3 times as long as wide 4. Sparse hairs on first antennae 5. Left first antenna of male thickened and hinged 6. Female with two egg sacs, the right sac containing only two eggs 7. Swims with gliding motion
Centropages typicus ♂ 1.0-1.60 mm ♀ 1.25-1.75 mm	1. Body rectangular with well defined head region 2. Female with large unequal "fenders" (right side larger) on posterior corners of fifth segment 3. Male with smaller unequal spines (left side larger) on posterior corners of fifth segment 4. Short hairs on first antennae First antennae reach beyond tips of caudal rami Tooth-like spines on the first, second, and fifth segments of male and female first antennae 5. Right first antenna of male thickened and hinged 6. No egg sacs present 7. Female genital segment with several stiff spines

Continued

APPENDIX 4-D (Continued)

DESCRIPTIVE CHARACTERISTICS OF SELECTED NERITIC COPEPODS

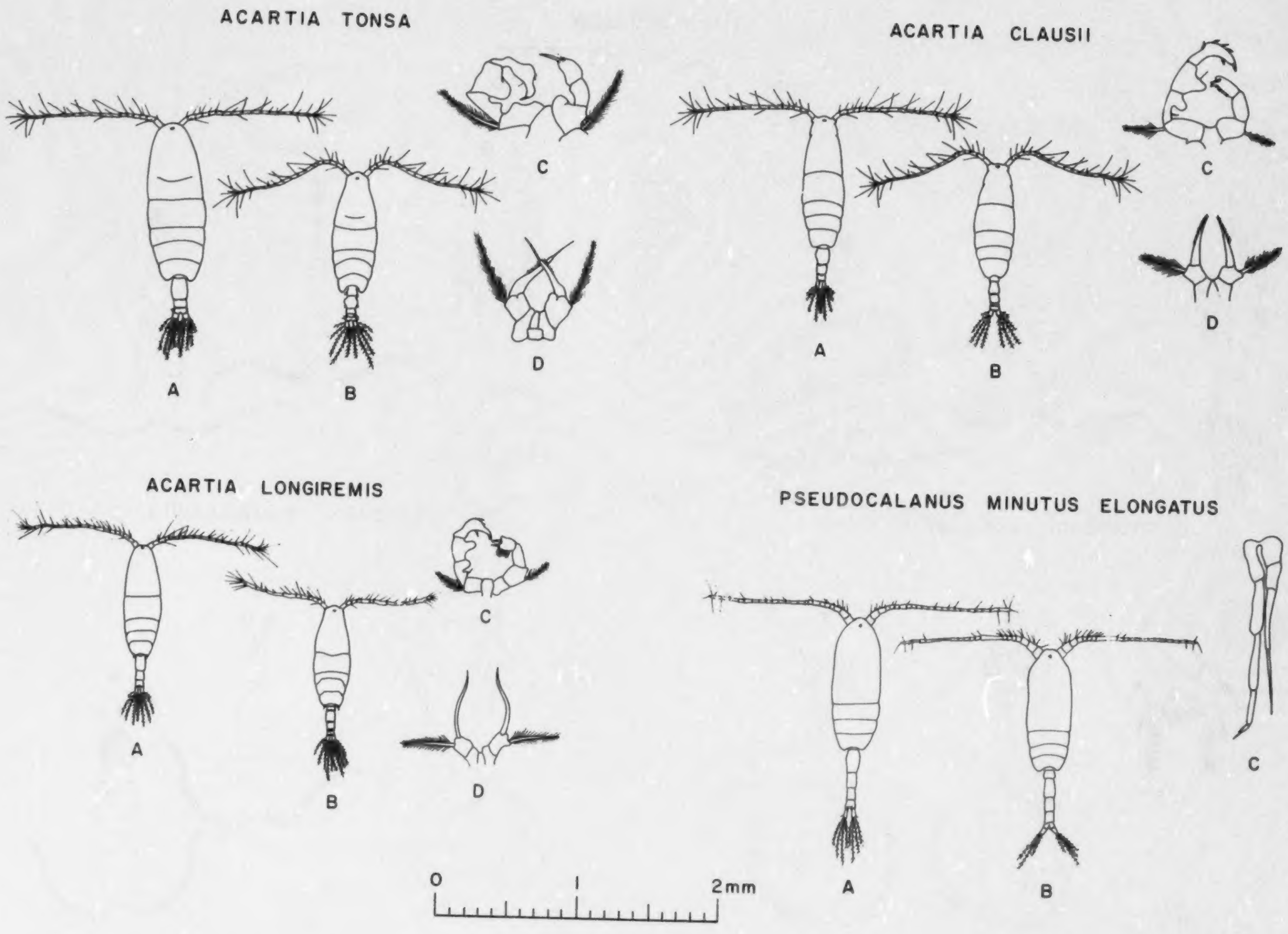
Species	Descriptive Characteristics
Centropages hamatus ♂ 0.9-1.2 mm ♀ 1.0-1.4 mm	1. Body rectangular with well defined head region 2. Female with unequal spines on posterior corners of fifth segment, the right turned outward 3. Male with symmetrical spines on posterior corners of fith segment 4. Short hairs on first antennae First antennae reach beyond tips of caudal rami No tooth-like processes present on first antennae 5. Right first antenna of male thickened and hinged but not as pronounced as in C. typicus 6. No egg sacs present
Tortanus discaudatus ♂ 1.75-2.00 mm ♀ 2.00-2.25 mm	1. Very large spindle-shaped body 2. Female has symmetrically curved spines on posterior corners of fifth segment 3. Urosome very asymmetrical in male and female 4. Female right caudal ramus twice as wide as left 5. Male caudal rami unequal 6. Male urosome curved to right 7. First antennae reach caudal rami 8. Right first antenna thickened and hinged in male 9. No egg sacs present

Continued

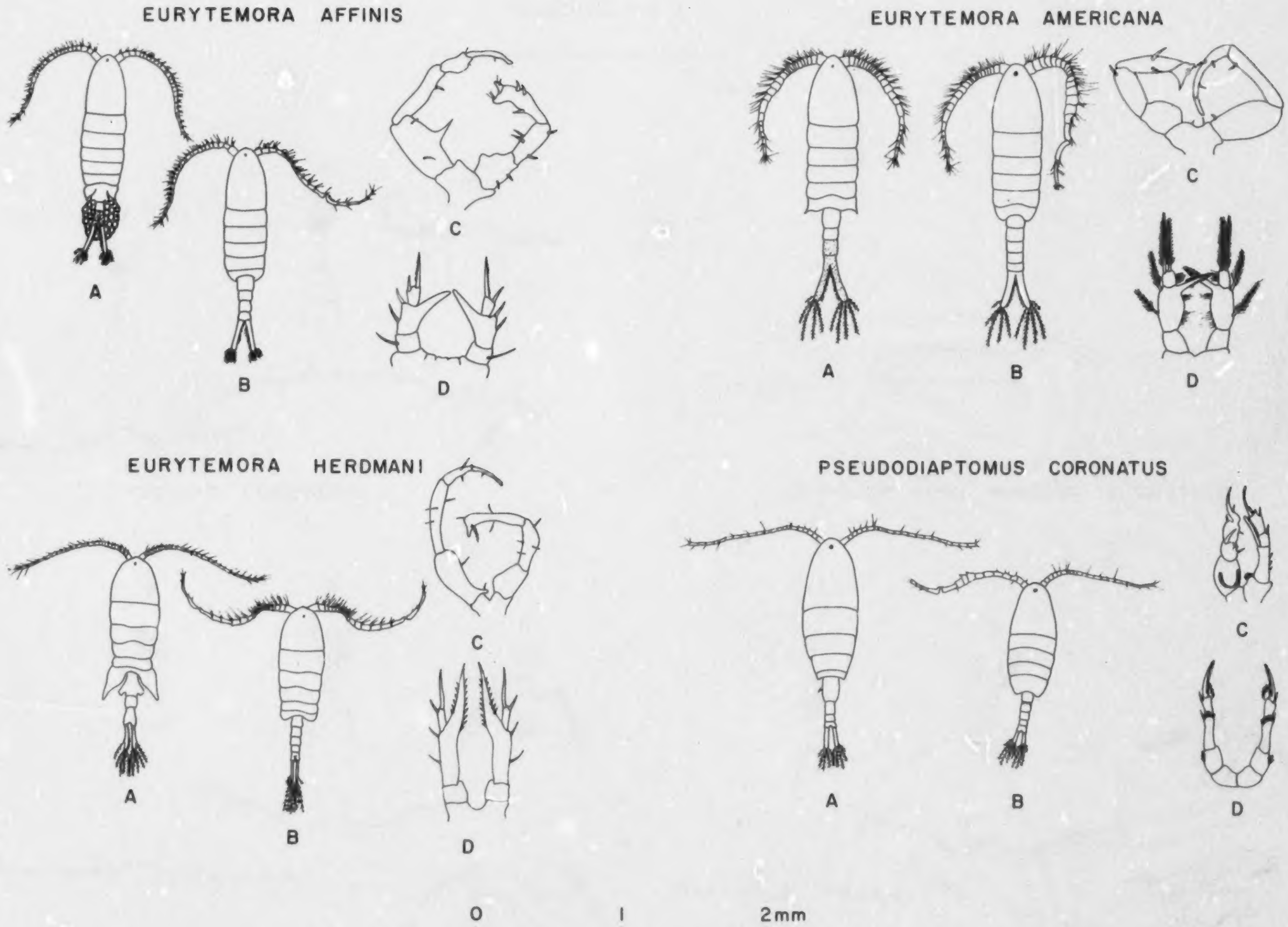
APPENDIX 4-D (Continued)

DESCRIPTIVE CHARACTRISTICS OF SELECTED NERITIC COPEPODS

Species	Descriptive Characteristics
Temora longicornis ♂ 1.00-1.35 mm ♀ 1.00-1.50 mm	1. Body shaped like bear's paw; wide at head, tapering rapidly to fifth segment 2. Caudal rami very long 3. Male urosome longer and narrower than female urosome 4. First antennae have very short hairs 5. Right antenna on male thickened and hinged 6. No egg sacs present
Oithona similis ♂ 0.6-0.70 mm ♀ 0.7-0.95 mm	1. Body spindle-shaped 2. Urosome 3/4 length of metasome 3. First antennae have very long hairs 4. First antennae of male hinged twice 5. Female has two ovisacs appressed to sides of urosome
Oithona nana ♂ 0.48-0.57 mm ♀ 0.50-0.65 mm	1. Spindle-shaped body 2. Urosome 3/4 length of metasome 3. First antennae have very long hairs 4. First antennae hinged twice on male



APPENDIX 4-D-1

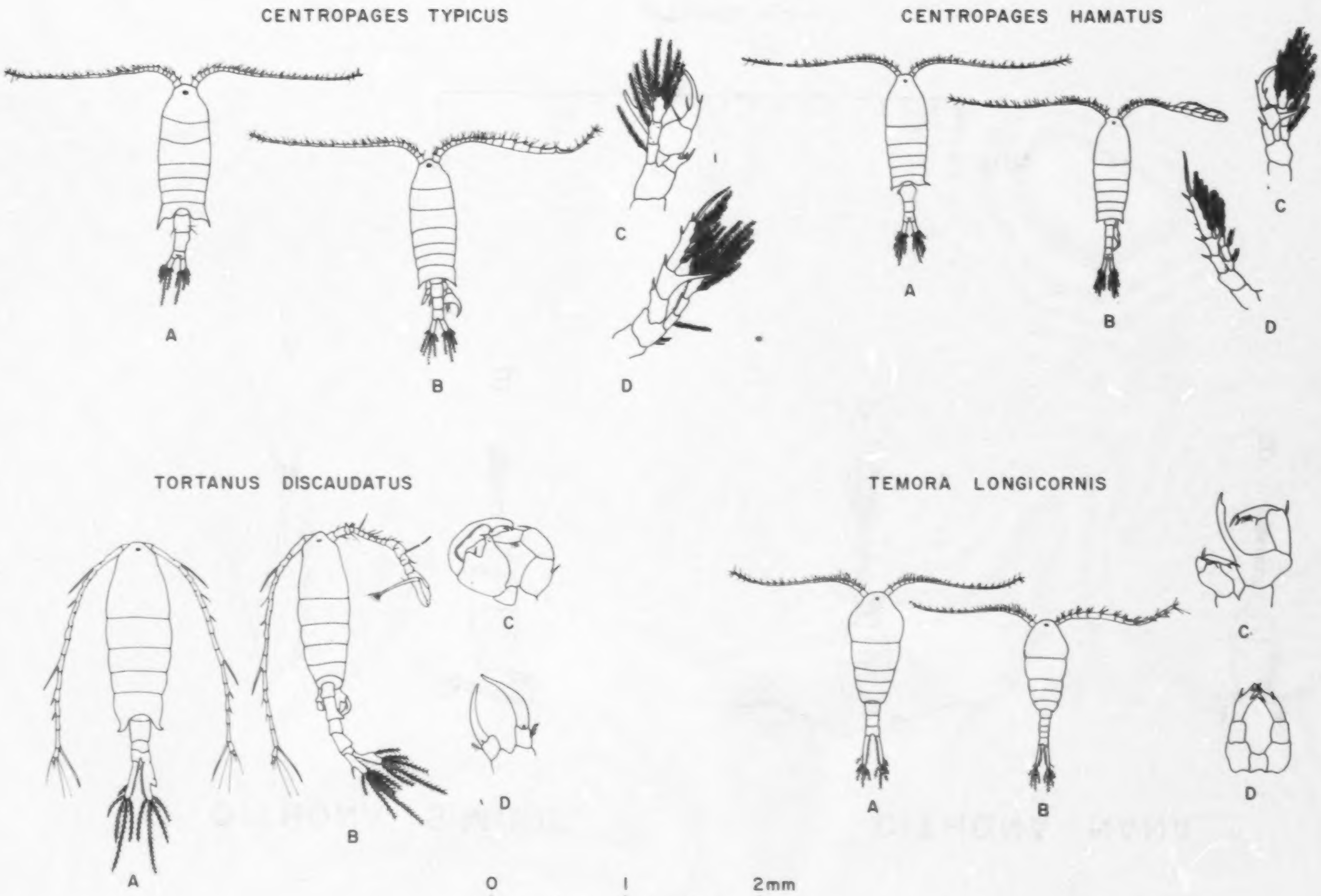

EURYTEMORA AFFINIS

EURYTEMORA AMERICANA

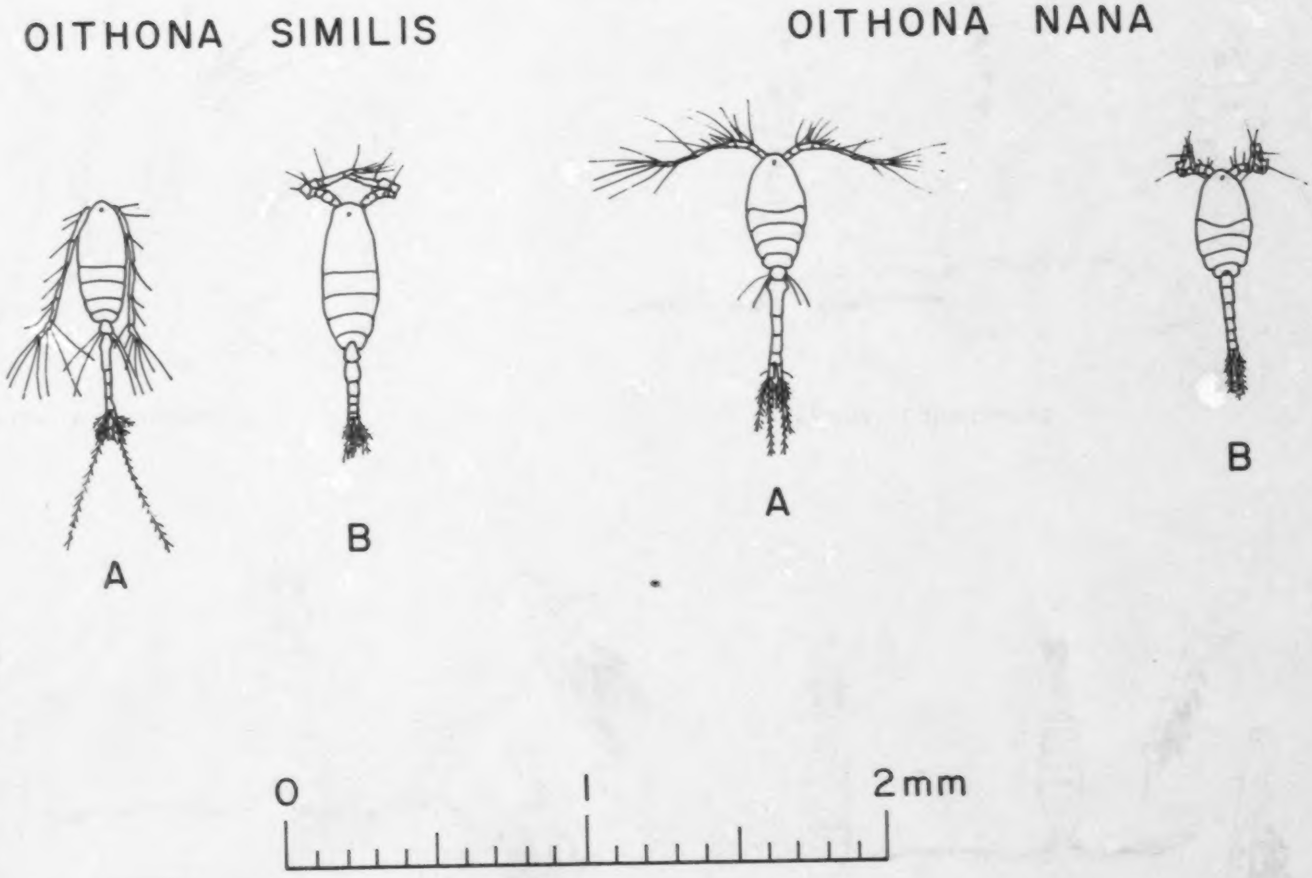
EURYTEMORA HERDMANI

PSEUDODIAPTOMUS CORONATUS

APPENDIX 4-D-2



APPENDIX 4-D-3



APPENDIX 4-D-4

REFERENCES

Katona, S.K. 1971. The Developmental Stages of *Eurytemora affinis* (Poppe, 1880)(Copepoda, Calanoida) Raised in Laboratory Cultures, including a Comparison with the Larvae of *Eurytemora americana* Williams, 1906, and *Eurytemora herdmani* Thompson and Scott, 1897. Crustaceana 21(1):5-20.

Eurytemora affinis: A, B, p.10, Fig. 47, 54, 49, 53 and p.14, Fig. 93, C, D, p. 13, Fig. 88, 85.

Mori, Takamochi. 1964. The Pelagic Copepoda from the Neighboring Waters of Japan. The Soyo Company, Inc. Tokyo, Japan. 150 pp. 80 pl.

Acartia clausi: B, Pl. 51, Fig. 9.
Acartia longiremis: B, Pl. 51, Fig. 6
C, Pl. 51, Fig. 9
Tortanus discaudatus: A, B, Pl. 52, Figs. 4, I.
Oithona similis: A, B, Pl. 62, Figs. 4, 8.
Oithona nana: A, B, Pl. 63, Figs. 1, 2

Sars, G.O. 1903. An Account of the Crustacea of Norway. Vol. IV. Copepoda. Bergen Museum. Alb. Cammermeyer's Forlag, Christiana. 171 pp. 108 pl.

Acartia longiremis: A, Pl. XCIX
Acartia clausi: C, Pl. CI
Pseudocalanus minutus elongatus: A, B, C
Pl. X, XI
Centropages typicus: A, B, Pl. XLIX, LI
Centropages hamatus: A, B, Pl. LII
Temora longicornis: A, B, Pl. LXV

Thompson and Scott. 1897. Proceedings, Liverpool Biological Society. Vol. XII, Pl. V p. 78.

Eurytemora herdmani: A, B, C, D, p. 78, Figs. 1, 2, 9, 11, 10, 8.

Wilson, Charles B. 1932. The Copepods of the Woods Hole Region Massachusetts. Smithsonian Institution. United States Government Printing Office. Bulletin 158. 635 pp.

Acartia tonsa: A, B, C, D, p. 161, Fig. 109 a, b, c, d.
Acartia clausi: A, D, p. 164, Fig. 112 a, b.
Acartia longiremis: D, p. 165, Fig. 113 c.
Eurytemora americana: A, B, C, D, p. 109, Fig. 72 a, b, c.
Pseudodiaptomus coronatus: A, B, C, D, p. 102, Fig. 68 a, c, b.

Centropages typicus: C, D, p. 88, Fig. 60 d, e.
Centropages hamatus: C, D, p. 89, Fig. 61 e, f.
Tortanus discaudatus: C, D, p. 167, Fig. 114 f, g.
Temora longicornis: B, C, D, p. 105, Fig. 70 b, d, e.

Acknowledgment: Special appreciation is extended to Ms. Lianne Armstrong for the illustrations in this appendix.

E. CULTURING THE MYSID (MYSIDOPSIS BAHIA) IN FLOWING SEA WATER OR A STATIC SYSTEM

D.R. Nimmo, T.L. Hamaker, and C.A. Sommers

INTRODUCTION

Many freshwater but few estuarine or marine animals have been found practical for life-cycle toxicity tests. Life cycles of certain marine species are complex: many require an estuarine existence as larvae or juveniles, followed by adult migration to deeper waters offshore to reproduce. Culture and maintenance of estuarine and marine species entail elaborate and expensive equipment with temperature or salinity controls, anticorrosion surfaces, and if necessary, special filtration systems. We have cultured the bay mysid, *Mysidopsis bahia*, for life-cycle toxicity tests at ERL, Gulf Breeze, in (1) flowing sea water and (2) a re-circulating aquarium. Both methods are described below; however, the re-circulating method is appropriate for laboratories not equipped with flowing seawater.

FLOWING SEA WATER METHOD

Mysids, collected from Santa Rosa Sound near Pensacola, Florida, are cultured in the laboratory in 38-liter glass aquaria supplied with filtered (20μ) flowing water (10 to 27 parts per thousand salinity) at 18 to 28°C. Mysids are fed 48-hour-old *Artemia salina* larvae daily. Overflow from each aquarium exits through a standpipe, where an attached ring of screen Nitex[R] prevents escape of mysids and *Artemia*. Thus, this species can be cultured continuously for 4-5 months without fluctuations in population density.

STATIC, RECIRCULATING METHOD

Although culture of mysids is more efficient in flowing water, less maintenance is required for cultures in the re-circulating aquaria. We maintained two cultures for 13 months in aquaria without changing the water. This method, now being refined, does not depend on large quantities of natural or artificial salt water. At least four aquaria are recommended to ensure sufficient production for continuing experiments. The most critical step in establishing a viable culture is the conditioning or aging of culture water.

[R] Nitex is a registered trademark of Tobler, Ernst, and Trabor, Inc., Murray St., New York, NY. Reference to commercial products does not constitute endorsement by the Environmental Protection Agency.

Physical System--

A 10-gallon (38-liter) glass aquarium, equipped with a Metaframe^{R} undergravel filter and Metaframe^{R} filter light, provides the basic system. Air-lift tubes are attached to each undergravel filter base to circulate water in the aquarium. To each air-lift tube, set in its filter base, is attached to a small glass chamber (vented into the atmosphere) mounted directly above the aquarium. This device collects air and water exiting from the airlift tube. Thus, air is vented and water is recycled immediately through a small glass tube directed toward the aquarium's center. Another option utilizes a u-tube attached to the air-lift tube to direct water and air downward toward the aquarium's center. The former design offers the advantage of an uninterrupted flow of water recirculated to the culture with minimal splash. Currents created by resultant water flow are necessary to orient adults especially during feeding.

Substratum--

Substratum can be either: *Coquina* sp. shell (mined in Florida for limited distribution) or Pilot^{R} brand crushed oyster shell (pullet size). For either substratum, 4.5 kilograms (10 lbs) are required per aquarium.

Culture Water--

If available, natural seawater is recommended; salinity must be adjusted to 22-26 ^{o}/oo with deionized water. The water, after filtered through a 20-micron filter, is added to the aquarium containing the shell. We substitute for natural sea water Rila^{R} marine mix salts which are autoclaved as a precaution against the presence of pathogenic microorganisms. *The water in the aquarium should be aged for at least 2-3 weeks before mysids are introduced*.

Water-Conditioning--

Water-conditioning is required, although no explanation can be offered concerning the changes that occur in quality of water. After adding water to each aquarium: (1) circulate the water, (2) illuminate the fluorescent light, and (3) introduce living biological material to facilitate the conditioning process. We shortened this process with these aids: algal mats from previous cultures; living, unfed *Artemia*. and mysids, if available. After mysids release their young and survive for 48 hours, the culture should be viable.

Food--

Mysids are fed *ad libitum* 48-hour-old *Artemia* nauplii. *M. bahia* is carnivorous, and, if food is not available, will cannibalize their young.

---

^{R}Metaframe Corporation, Elmwood Park, NJ 07407 and Comptom. CA 90220.

^{R}Pilot brand is a registered trademark of Oyster Shell Products, subsidiary of Southern Industries Corp., Mobile, AL; Houston, TX; Baltimore, MD.

^{R}Rila is a registered trademark of Rila Products, P.O. Box 114, Teaneck, NJ

F. METHODS FOR ACUTE STATIC TOXICITY TESTS WITH MYSID SHRIMP (MYSIDOPSIS BAHIA)

Patrick W. Borthwick

INTRODUCTION

Mysidopsis bahia is a shrimp-like estuarine crustacean that has been shown to be very sensitive to toxic substances and used successfully in acute static toxicity tests with complex wastes. M. bahia is recommended as a test species due to its sensitivity, short life-cycle, small size, and practical culture methods (Nimmo et al., 1977 and 1978). Results from toxicity tests with mysids can be used to estimate the impact of ocean-dumped materials on other salt water crustaceans.

SELECTION OF TEST CONTAINERS

Based on comparable toxicity tests with mysids exposed in different containers, control survival is best when glass 2-liter Carolina[R] dishes are used instead of 4-liter, wide-mouthed jars. These cylindrical, stackable culture dishes provide a large surface-to-volume ratio and ample horizontal space to minimize cannibalism. When filled with test medium to 1 liter, culture dishes allow easy visual examination of the mysids. Observation is hampered if the test medium is turbid or dark. Stocking density should not exceed 10 mysids/liter to insure minimal loading and ease of counting. Culture dishes may be stacked and placed in a temperature-controlled incubator. Minimal disturbance and continual lighting help prevent mysids from "jumping out" of the test medium and "sticking" to the sides of the test container. If evaporation is evident (especially at high temperatures), distilled or deionized water should be added to the test medium daily to prevent hypersaline conditions.

SALT WATER AND TEST MEDIA PREPARATION

Materials considered for ocean disposal vary in solubility and complexity. Thus, several approaches are necessary for testing various types of wastes.

Effluents from ocean outfalls may contain a mixture of wastes that are substantially diluted with fresh water. To achieve a desired salinity in the test medium without further diluting the effluent, it is necessary to add dry, autoclaved artificial sea salts to the effluent. This is best accomplished by stirring the effluent with a magnetic stirrer while the dry salt mix dissolves.

[R] Obtained from Carolina Biological Supply Co., Burlington, NC 27215

Materials tested for ocean disposal (e.g. liquid, solid, concentrate, or sludge) should be diluted or suspended with filtered natural sea water that can be adjusted to a desired salinity by adding artificial salts or deionized or distilled water.

If soluble, single compounds, or substances of relatively simple or known composition, should be added directly to the test solution. Insoluble materials should be tested as a suspension, rather than with a carrier, to solubilize test material. Filtered natural sea water, if available, should be used in lieu of artificial sea water, particularly if the mysids were cultured in natural sea water.

Test media are prepared, stirred to uniformity, and allowed to equilibrate to the test temperature for at least 30 minutes before test animals are introduced.

CARE AND HANDLING

Mysids are easily mishandled; special care in transferring animals from cultures to culture dishes is mandatory.

Mysids are removed from cultures with a glass tube (300 x 9.0 mm i.d.), fire-polished at both ends. By placing the index finger over the end of the empty tube and submersing the tip, a single mysid can be captured gently by breaking the finger seal. Then the mysid can enter the tube in surrounding water. Ten newly hatched individuals are assigned randomly to a series of 30 mℓ glass beakers containing sea water. Sea water volume in each beaker is reduced to 5 mℓ, and a beaker containing ten mysids is gently submersed into each culture dish until the test animals swim into the test medium. It is often difficult to remove a beaker that has no mysids on the glass surface. Therefore, beakers must be carefully inspected and the number of mysids confirmed in each test container. Mysids are handled, observed, and counted over a waterproof lighted table; sudden movements or disturbances must be avoided.

SELECTION OF TEST ANIMALS

Newly hatched juvenile mysids ($\leq$24-hour-old) are used because of their uniform size and proven success in toxicity tests. Test results are confounded if brooding mysid females release young into the test medium--thus affecting loading, uptake of toxicant, and competition for food and space. To obtain juveniles, isolate several brooding females in a large beaker the day before the test, and harvest the young on the day of the test. Care in handling is essential to a successful toxicity test.

FEEDING

Mysids have a short lifecycle, and their metabolic demands are high. They seem to thrive best when fed living 48-hour-old Artemia nauplii. For 96-hour acute static toxicity tests, I recommend that mysids be fed 10 to 20 nauplii per mysid per day to minimize cannibalism. Although it is generally undesirable to feed most fish and macroinvertebrates during static toxicity tests, an exception is necessary for M. bahia.

EXPERIMENTAL DESIGN

The recommended test procedure for 96-hour acute static toxicity tests with mysids should include a sea water control, carrier control (if applicable), and at least five concentrations of test media. When materials of unknown toxicity are tested, a range-finding test may be necessary to approximate the range of concentrations for the definitive test. Two replicate tests of 10 mysids each are desirable for each concentration in definitive tests. Animals should be randomly assigned to the culture dish test containers 30 minutes after 1 liter of medium is added. For additional details, consult the section, "Static Method for Acute Toxicity Tests Using Fish and Macroinvertebrates."

OBSERVATIONS

Mysids are observed at 24, 48, 72, and 96 hours to determine the number of dead or affected individuals. Dead animals should be removed when observed. Observations should note erratic swimming, loss of reflex, molting, cannibalism, unusual behavior, discoloration, and ability of individuals to capture live Artemia during feeding.

CALCULATIONS AND REPORTING

For definitive tests, the 96-hour LC50 and 95-percent confidence limits must be calculated, using Probit Analysis (Finney, 1971). For range-finding data, the LC50 can be estimated by linear interpolation.

Reports should follow the outline in the section titled, "Static Method for Acute Toxicity Tests Using Fish and Macroinvertebrates."

REFERENCES

Finney, D.J. 1971. Probit Analysis, 3rd. ed., Cambridge Univ. Press, London and New York.

Nimmo, D.R., L.H. Bahner, R.A. Rigby, J.M. Sheppard, and A.J. Wilson, Jr. "Mysidopsis bahia: an Estuarine Species Suitable for Life-Cycle Toxicity Test to Determine the Effects of a Pollutant," Aquatic Toxicology and Hazard Evaluation, ASTM STP 634; F.L. Mayer and J.L. Hamelink, Eds., American Society for Testing Materials, 1977, pp. 109-116.

Nimmo, D.R., R.A. Rigby, L.H. Bahner, and Jim Sheppard. 1978. Acute and Chronic Effects of Cadmium on the Estuarine Mysid, Mysidopsis bahia, Bull. Env. Contam. Toxicol. 19(1) (in press).

G. ENTIRE LIFE CYCLE TOXICITY TEST USING MYSIDS (*MYSIDOPSIS BAHIA*) IN FLOWING WATER

D.R. Nimmo, T.L. Hamaker, and C.A. Sommers

INTRODUCTION

The purpose of this method is to determine effects of continuous exposure of a pollutant on the survival, reproduction, growth, and behavior of this crustacean through a life cycle. Among the advantages of using this species in toxicity tests are: (1) ease of culture and maintenance; (2) short generation time (14-17 days depending on the temperature); and reproduction data based on actual count (of juveniles) rather than estimates. Further, this species is representative of many intermediates in estuarine food webs. Data on toxicity, reproduction, and growth, using a modification of the procedure described here, have been published (Nimmo et al., 1977).

Mysidopsis bahia is an estuarine species and three reports in the literature (Molenock, 1969; Odum, 1972; Nimmo et al., 1977) suggest that its range is from Galveston, Texas to Miami, Florida. We have successfully captured mysids from small shallow ponds fed by salt water from Santa Rosa Sound near Pensacola, Florida. A small fish net, used by tropical fish retailers, or a 3-4 foot push net with very small mesh, is sufficient to capture the adult mysid shrimp.

PHYSICAL SYSTEMS

Test Water--

1. The source of test water should be a natural water with a salinity ≥15 °/oo, although mysids can live at much lower salinities. We have observed that mysids have survived for 72 hours at 2-3 °/oo but reproduction is affected at 6-8 °/oo if maintained for prolonged periods.

2. Sea water must be filtered to remove particles 15μ or larger to remove planktonic larvae that grow, then prey on mysids or their food during the test.

3. The water source must be analyzed for pollutants such as pesticides, PCB's, and metals. Special determinations should be made for those chemicals being investigated in the toxicity tests.

Dosing Apparatus--

We suggest that all tests be conducted in intermittent flows from a diluter or in continuous flow with the toxicant added by an infusion pump. Further, we recommend the procedures of Mount and Brungs (1967) or Hansen et al. (1974) if the toxicant can be added without a solvent; the device described by Hansen et al. (1974) if a solvent is necessary; or procedure of Bahner et al. (1975) if pumps are required for continuous flow.

Aquaria--

Glass aquaria 34 x 72 x 18 cm with a water depth of 6 cm are preferred, but we have used smaller aquaria (12 x 24 x 12 cm) with good results. When each aquarium receives the maximum volume of salt water from a diluter or continuous flow apparatus, a self-starting siphon reduces the volume to about one liter. Therefore, water levels, fluctuating at intervals of about 30 min, ensure an exchange of salt water within each aquarium and the small chambers devised to retain the mysids.

Retention Chambers--

The chambers consist of a standard, 10-cm glass petri dish (or cover) to which a 15-cm-high cylinder of Nitex^R screen (mesh number 210) is attached by silicone cement.

Test Procedures

Flow Rate of Test Water--

Flow rates to each aquarium should (1) provide 90 percent replacement in 8-12 hours (Sprague, 1969); (2) maintain dissolved oxygen 60 °/o saturation; and (3) maintain the toxicant concentration. Our flow rate is 25ℓ/hour/test aquarium for the continuous-flow system; for the diluter, 6ℓ/hour/test aquarium. We suggest that dissolved oxygen determinations be made twice weekly.

Lighting--

Lighting is continuous, using flourescent bulbs.

Temperature--

Test temperatures should be maintained above 20°C, although we have conducted a test successfully with temperatures above 15°C. We maintain temperature > 20° and < 30°C by heating or cooling procedures.

Cleaning and Aeration--

We do not clean any test chambers used to retain the animals during the test. Instead, we transfer mysids to a pre-cleaned chamber. For aeration, a small stream of compressed air is delivered into each chamber to safeguard against possible anoxic conditions and to create a current that apparently aids animal orientation.

---

^{R}Nitex is a registered trademark of Tobler, Ernst and Trabor, Inc., Murray St., New York, NY Reference to commercial products does not constitute endorsement by the Environmental Protection Agency.

e. Concentrations of Toxicants

(1) When we use a diluter to deliver the toxicants, we use a minimum of four concentrations of the toxicant and a control with carrier; and a carrier control, a control without carrier, and four concentrations of toxicant for the continuous flow system.

(2) In many instances, a carrier is necessary to disperse and dissolve the toxicant in the test water. Therefore, we employ either acetone or triethylene glycol (Bahner et al., 1975).

(3) Concentrations selected must adversely affect at least one, but not all life stages of the mysid and this is usually noted as death. Concentrations for chronic toxicity tests should be based on results of acute flow-through toxicity tests. Selection of test concentrations is difficult because chronic effects on survival, growth, or reproduction of mysids can occur at concentrations that range from 0.5 to 0.0001 of the 96-hour LC50. The accuracy of the selection process can be improved by some preliminary tests such as (a) acute, 96-hour, flow-through tests using different life stages (e.g. adult, juvenile) and (b) acute tests to determine incipient LC50 (Sprague, 1969). The highest concentration in life-cycle tests generally should be the lowest concentration affecting survival or growth in preliminary tests.

(4) The material and water should be analyzed in this test. Water from each aquarium should be analyzed at least twice during the 96-hour test; water samples should also be analyzed from one duplicate weekly during the life-cycle test. Cost and complexity of analyses, as well as conclusions and decisions based on test results, should dictate frequency and number of samples.

Test Procedure--

At the outset, we isolate 250 to 350 gravid female mysids in a 5-liter glass battery jar and allow an extremely slow flow of salt water (about 4 drops/second) to drip into the jar. The outflow exit is an automatic siphon, whose inlet is covered by Nitex screen (see retaining chambers). We maintain a constant supply of Artemia nauplii to mysids for 24 hours and remove juvenile mysids about every 3-4 hours until their number is sufficient to begin a test. Thus, we have a synchronous population (within 24 hours of age) of mysids for our tests. We begin the test with eight retention chambers, five juveniles each or 40 animals per concentration.

Food--

All mysids in the retaining chambers are fed 48-hour-old Artemia nauplii ad libitum daily.

Test Progression and the F_1 Generation--

(1) In monitoring daily changes in survival or populations, the retaining chamber is lifted gently from the aquaria, water is drained from the

Nitex cylinder to the level of the Petri dish, and the chamber is placed on a lighted counter top. We record live animals by sex, number, females with or without brood pouches, their young, and any other germane criteria. Dead mysids are removed.

(2) On days 8-12, mysids are categorized by sex and number per retention chamber. Juveniles which are usually released beginning on day 14 are counted and removed.

(3) Our life cycle test can be completed in 12 days by maintaining the temperature at 29°C, but we recommend a test temperature between 22° and 25°C, if possible; otherwise, $\geq$20° and $\leq$30°C. We also recommend that testing continues for 26-28 days. Recommended time and temperature allow the females tested to complete multiple broods (about three broods with number of juveniles varying per brood); thus, number of young per female or the reproductive success is more easily observed. On the basis of seven toxicants tested to date, reproductive success (number of juveniles per female) is the most consistent criterion of sublethal effects; the pesticide Kepone, however, affected growth of females at a lower concentration.

(4) To test whether the F_1 generation is susceptible to the toxicant, we isolate 20 juveniles in separate chambers (5 per chamber) and follow development of these animals until the onset of reproduction (F_2).

Statistical Analysis

The LC50's and the 95% fiducial limits are calculated by linear regression analysis after probit transformation. We employ Dunnett's "t" test, comparing mean brood size (number of young per female) in multiple treatments to control. Data such as growth, determined by measurement of length, may be amenable to analysis of variance, or chi-square tests. We use $\alpha \leq 0.05$ as significant difference.

REFERENCES

Bahner, L.H., C.D. Craft, and D.R. Nimmo. 1975. A Saltwater Flow-through Bioassay Method with Controlled Temperature and Salinity. Progressive Fish-Culturist 37:126-129.

Hansen, D.J., S.C. Schimmel, and J. Forester. 1974. Aroclor 1254 in Eggs of Sheepshead Minnows (Cyprinodon variegatus). Effect of Fertilization Success and Survival of Embryos and Fry. Proc. 27th Ann. Conf. Southeast. Assoc. Game Fish Comm. Oct. 1973. Hot Springs, Arkansas: 420-426.

Hansen, D.J., P.R. Parrish, J.I. Lowe, A.J. Wilson, Jr., and P.D. Wilson. 1971. Chronic Toxicity, Uptake, and Rentention of Aroclor^{R} 1254 in Two Estuarine Fishes. Bull. Environ. Contam. Toxicol. 6:113-119.

Molenock, J. 1969. Mysidopsis bahia, New Species of Mysid (Crustacea: Mysidacea) from Galveston Bay, Texas. Tulane Studies in Zoology and Botany, 15(3):113-116.

Mount, Donald I., and William Brungs. 1967. A Simplified Dosing Apparatus for Fish Toxicology Studies. Water Research 2:21-29.

Nimmo, D.R., L.H. Bahner, R.A. Rigby, J.M. Sheppard, and A.J. Wilson, Jr. 1977. Mysidopsis bahia: an Estuarine Species Suitable for Life-cycle Toxicity Tests to Determine the Effects of a Pollutant. F.L. Mayer and J.L. Hamelink, Eds., American Society for Testing and Materials STP 634:109-116.

Odum, W.E., and E.J. Heald. 1972. Trophic Analysis of an Estuarine Mangrove Community. Bull. Mar. Sci. Gulf and Caribbean 22(3):671-738.

Sprague, J.B. 1969. Review Paper: Measurement of Toxicity to Fish. 1. Bioassay Methods for Acute Toxicity. Water Research 3(11):793-821.

H. CULTURE OF THE GRASS SHRIMP (*PALAEMONETES PUGIO*) IN THE LABORATORY

Dana Beth Tyler-Schroeder

The grass shrimp, *Palaemonetes pugio*, a useful organism in assessing toxicity of various materials, is (1) easily cultured in the laboratory, (2) sensitive to toxicants, and (3) can be exposed in flow-through systems throughout a life cycle. Culture and holding procedures for the grass shrimp are described below.

INDUCTION OF SPAWNING

Laboratory spawning of *P. pugio* was first described by Little (1968). Deposition of eggs began five to eight weeks after initiation of a photoperiod and temperature regime. Egg production is directly proportional to rostrum-telson length of females greater than 18 to 20 mm (Jensen, 1958; Wood, 1967). Shrimp are sexed by examination of the second pleopod (Meehean, 1936), but field data show a 50/50 ratio of sexes (Wood, 1967).

I have found that spawning can be induced at a constant temperature of 25°C, or above, with appropriate increase in photoperiod. A minimum photoperiod of 10 hours light:14 hours darkness per day at 25°C is necessary to activate ovarian development and spawning. Egg deposition usually follows within two to four weeks after this regime is established. Continued spawning of laboratory populations has been observed when the light portion of the photoperiod is increased by a 47-minute increment every one or two weeks until the photoperiod is 15 hr 29 min light, and 8 hr 31 min dark.

Ovarian growth and egg deposition can be accomplished with 100-watt, 1750-lumen incandescent light bulbs, as well as fluorescent and growth-light lamps. However, the latter types stimulate undesirable algal growth in tanks, a feature to be avoided in toxicity exposures.

Although spawning can be successfully accomplished in non-recirculating aquaria, the time period can be shortened in flow-through aquaria. A change of water in static aquaria containing conditioned shrimp is followed by a burst of egg deposition. Therefore, I postulate that shrimp produce a substance that inhibits spawning in overcrowded, stagnant conditions. Thus, I recommend that spawning in static systems be accomplished by (1) using a recirculating system with a biological filter, and/or, (2) changing the water at least weekly. Shrimp being conditioned for spawning must be fed a daily diet of freshly hatched *Artemia* nauplii and a commercial fish flake food containing vegetable and animal material, but no detectable pollutants.

Laboratory egg production is similar to natural production: each female deposits from 100 to 500 eggs each spawning and may spawn every four to six weeks. Incubation of larvae on the pleopods of the female requires from two to three weeks. Release of larvae frequently is followed by deposition of a new egg mass.

LARVAE PRODUCTION

To produce a number of larvae for toxicity testing, rearing, etc., transfer a number of ovigerous females from the spawning population to a hatching apparatus. Larvae may be hatched under static conditions as described in the section "Static Bioassay Procedure Using Grass Shrimp (Palaemonetes sp.) Larvae" or in the flow-through system described here.

Shrimp can be cultured in a hatching apparatus (Figure 1) using filtered, flowing seawater, and two commercially available 37.8-liter (10 gal) aquaria.

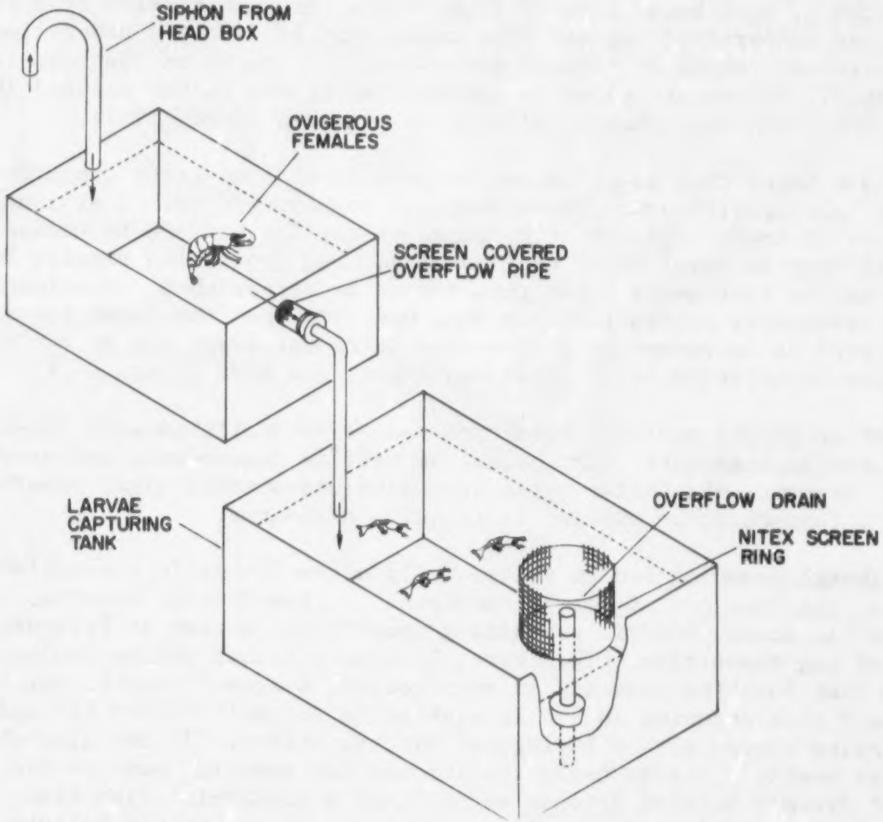


Figure 1-H A flow-through hatching apparatus for grass shrimp larvae production.

Filtered seawater (20 μm) is introduced into the first aquarium by calibrated siphon at a flow-rate of approximately 1 liter/hour. The water is heated to 25°C by a small aquarium heater. Approximately 50 to 75 ovigerous females are placed in the first aquarium. Chelipeds must be removed with a pair of fine, surgical scissors to reduce removal of the eggs by the females. They are fed freshly hatched Artemia nauplii daily. The overflow drain (35 mm diameter) from the first aquarium is covered with a nylon mesh screen (2,000 μm) to prevent loss of adult females.

Newly hatched grass shrimp larvae pass through the overflow from the first aquarium containing ovigerous females into a second aquarium fitted with a special drain to retain the larvae. The drain pipe consists of a neoprene stopper bored to hold a length of 10- to 12-mm glass tubing. The length of the glass tubing determines water level in the larval tank. A disc of plexiglass on which is cemented a collar of nylon mesh screen (363μm) is fitted on the glass tubing. The nylon collar is of sufficient mesh size to prevent larvae from being flushed from the tank. This collar must extend one or two cm above water level to prevent loss of larvae due to unexpected change of water depth in the larval aquarium.

Larvae are attracted to a lamp placed at one corner of the tank and then collected daily. They may be removed by a wide-mouth pipette or a small piece of nylon screen (363 μm). To obtain larvae of uniform age, drain and flush the larvae capturing aquarium with freshwater, refill with saltwater, and collect larvae the next day. Uniform age of larvae is frequently an important consideration in toxicity testing and culture.

REARING TO SEXUAL MATURITY

Grass shrimp larvae can be reared from larvae to adulthood in the laboratory. Newly hatched larvae can be reared in 90-liter (23.7 gal) aquaria (83 cm long x 41 cm wide x 35.5 cm deep). Water depth can be maintained at 30 to 32 cm by using a nylon mesh covered drain as described for the larval aquarium of the hatching apparatus. Temperature-salinity optima for P. pugio larvae, 25°C-25^{o}/oo (Floyd, 1977), should be maintained.

We have had success with stocking densities from 20 to 33 larvae/liter and water flow rates of 1 liter/hour, using calibrated siphons and constant head boxes. A slow flow rate through the aquaria allows a slower turnover of food organisms (freshly hatched Artemia nauplii), thus enhancing survival of the grass shrimp larvae. Maintain dissolved oxygen by use of a small aquarium air pump and one or two airstones.

Metamorphosis to postlarvae occurs within 12 to 35 days after hatching; thereafter, it is usually advisable to remove postlarvae to less crowded conditions, i.e., a larger aquarium (51 cm long x 67.5 cm wide x 24 cm deep). Grass shrimp juveniles can be reared to adulthood on a diet of live Artemia nauplii, frozen adult Artemia, or fish flake food. Sexual maturity should be achieved in one to two months after larvae hatch.

REFERENCES

Floyd, W.R. 1977. The Effects of Temperature and Salinity on the Larval Development of the Grass Shrimp, Palaemonetes pugio Holthuis, Reared in the Laboratory. Master's Thesis, Old Dominion University, Dept. of Oceanography, Norfolk, VA. 145 p.

Jensen, Jens Peder. 1958. The Relation Between Body Size and Number of Eggs in Marine Malacostrakes. Meddelelser Fra Danmarks Fiskeri-Og Havundersogelser. 2(19):1-25.

Little, Georgiandra. 1968. Induced Winter Breeding and Larval Development in the Shrimp, Palaemonetes pugio Holthuis (Caridea, Palamonidae). Studies on Decapod Larval Development, Supplement 2. Crustaceana: 19-26.

Meehean, O. Lloyd. 1936. Notes on the Freshwater Shrimp, Palaemonetes paludosa (Gibbes). Trans. Amer. Micros. Soc. 55:433-441.

Wood, Carl E. 1967. Physioecology of the Grass Shrimp, Palaemonetes pugio, in the Galveston Bay Estuarine System. Contr. Mar. Sci. Univ. Tex. 12:54-79.

I. STATIC BIOASSAY PROCEDURE USING GRASS SHRIMP (*Palaemonetes* SP.) LARVAE

D. B. Tyler-Schroeder

INTRODUCTION

Procedures for static 96-hour bioassays utilizing grass shrimp larvae, *Palaemonetes* sp., are outlined here. The grass shrimp is an obvious bioassay choice for several reasons. Three species of the genus, *P. pugio*, *vulgaris*, and *intermedius*, are common inhabitants of estuaries along the Gulf and Atlantic coasts of the United States (Holthuis, 1949, 1952). They are easy to collect and maintain in the laboratory. Field populations are usually quite large, allowing greater numbers to be brought into the laboratory for testing. By manipulating environmental conditions of temperature and photoperiod, it has been possible to induce spawning in the laboratory (Little, 1967), opening the way to laboratory cultures of genetic uniformity. Developing larvae are also available throughout the year for testing with these methods.

Larval stages of the three species are hardy and easy to culture in the laboratory. Developmental stages have been described for all species (Broad, 1957a, b; Broad and Hubschman, 1962; Hubschman and Broad, 1974), and salinity-temperature optima are known for the larval development of *P. vulgaris* (Sandifer, 1973). Developing larvae have demonstrated a susceptibility to polychlorinated hydrocarbons greater than that demonstrated by adults or juveniles (Tyler-Schroeder, unpublished manuscript).

CULTURE METHODS

Palaemonetes sp. are easily collected from the field with dip nets or seines in grassy, shallow estuarine areas. They can also be reared in enclosed holding ponds.

To obtain larvae, 8" glass culture bowls, such as the Carolina[R] culture dish, containing 1ℓ of filtered sea water are stocked with 3 ovigerous female shrimp per bowl. In order to produce enough shrimp larvae for a 96-hour test series (210 per replicate, 630 per test series), at least 17-25 bowls of ovigerous females (51-75 shrimp) must be maintained continuously in the laboratory (Figures 1-I and 2-I). The species of each female is

---

[R] Carolina Biological Supply Company, Burlington, North Carolina 27215. Mention of commercial products or trade names does not constitute endorsement by the Environmental Protection Agency.

Concentration(s) (mg/liter - ppm)	Control	0.01	0.1	1.0	10
Number larvae per test container	30	30	30	30	30
Total $= \frac{\text{150 larvae}}{\text{Replicate}}$					
Larval age and Number of replicates			1st Day larvae 3 replicates 18 Day larvae 3 replicates		
	(150 larvae/replicates) X (3 replicates/test) - 450 larvae (450 larvae/test) X (2 test ages) = 900 larvae				
Total number of larvae	Total $= \frac{\text{900 larvae}}{\text{Test Series}}$				
Example mortality:					
%	0	3	10	80	97
ppm	Control	0.01	0.1	1.0	10.0

Estimated LC50 between 0.1 and 1.0 ppm

Figure 1-I. Example of a range-finding bioassay.

Concentrations (ppm) (chosen from range-finding tests, Figure 1-E)	Control	0.1	0.159	0.252	0.399	0.631	1.0
Number of larvae per test concentration	30	30	30	30	30	30	30
Larval age and number of replicates				1st Day larvae 3 replicates			
				18th Day postlarvae 3 replicates			

Total number (210 larvae/replicate) X (3 replicates/test) = 630 larvae

Larvae (630 larvae) X (3 test ages) = 1890 larvae

Total $= \frac{\text{1890 larvae}}{\text{Test Series}}$

Figure 2-I. Example of a definitive bioassay.

confirmed and the chelipeds removed with fine surgical scissors to prevent removal of the eggs by the females. Shrimp in culture bowls are fed Artemia nauplii daily, and water is changed if a slight cloudiness appears. Since eggs are carried for 2-3 weeks before hatching, it is advisable to select females with eggs in the more advanced stages of development.

Larvae are removed from bowls containing ovigerous females each morning and mixed together to insure uniformity of test animals. They are randomly dispensed into 8" glass culture bowls containing 1ℓ of filtered sea water (200 larvae/ℓ), fed Artemia nauplii, and reared to the desired test age. Food is added daily and water changed when a slight cloudiness appears. There should always be sufficient live food in rearing and test chambers, since insufficient food accentuates developmental variability (Broad, 1957b) and produces undesirable variation in test results.

A 10 to 15% mortality must be anticipated in calculating the number of test larvae that must be reared to a predetermined age. Ideally, the larvae to be used in a series of 96-hour acute tests should be hatched at one time and reared in mass culture. Samples of larvae would be removed from this culture at designated times for testing. This technique would minimize or circumvent problems due to possible seasonal variation in larval susceptibility to waste material.

Salinity-temperature optima for P. vulgaris larvae indicate a broad range of tolerance to environmental conditions, which is most likely true for P. pugio and P. intermedius. Survival of P. pugio is approximately the same when reared in the laboratory at a temperature of 25°C and salinities of from 15-25 ^{o}/oo (A.N. Sastry, personal communication*). Bioassays should be performed within this range, preferably closer to 15 ^{o}/oo salinity, as P. pugio taken from the field are most commonly found in this salinity, or lower.

Preparation of Test Media, Selection of Test Containers

The nature of the material to be tested indicates choice of test container size and shape, preparation of test concentrations, and frequency of test media replacements. Problems posed by various wastes include insolubility in sea water, adsorption to exposed surfaces, decomposition by hydrolysis, photolysis, etc., loss by volatilization, high BOD, and bacterial growth. Such problems can affect results by causing variation from the calculated concentration of waste being tested, changing pH of test medium, releasing breakdown products which may be more or less toxic than parent compounds, and causing test animal mortality not related to direct effect of toxicants. Glass is the recommended material for test containers.

---

*A.N. Sastry, Graduate School of Oceanography, University of Rhode Island, Kingston, Rhode Island 02881

When choosing container size, it is important to choose a small vessel surface area to volume ratio because of possibility of adsorption. A larger volume is also important because of stocking density requirements. The 8" diameter, Carolina culture dish, containing 1ℓ of media, has been found to be a satisfactory test container for bioassay of Palaemonetes larvae, allowing maximum volume per vessel surface area and an acceptable stocking density of 30 larvae/ℓ.

Test media should be prepared fresh at the time of replacement, so that decomposition of toxicant, adsorption to preparation containers, depletion of oxygen, and bacterial growth are minimal. Likewise, it is necessary to change solutions in test containers at least every 24 hours, preferably every 12 hours.

All sea water to be used should be of natural origin, preferably from the dumping site. It should be filtered through a filter of 1μ porosity. To adjust salinity the addition of either distilled water or a high-salinity brine is necessary. The high-salinity brine may be of natural or artificial origin. If natural origin is desired, place a closed container one-half to three-quarters full of filtered sea water (≥30 ^{o}/oo salinity) in a freezer until solid throughout, usually 2-3 days. Subsequent to removal from the freezer, the supernatant is drained after the first thaw (2-3 hours). Supernatant should be 80-110 ^{o}/oo salinity or above and can be stored indefinitely.

An artificial brine may be made with any of the commercial artificial sea salts and distilled water, but should be used with caution. Several of these preparations contain one or more chelator substances, e.g., EDTA, which would bias test results with waste material containing heavy metals. The use of artificial sea water totally in place of natural sea water is not recommended at this time. In addition to various chelators in commercial preparation, the presence of high levels of contaminant heavy metals in artificial or laboratory prepared sea salt mixes should be checked. Several shelf chemicals are known to have background levels of Cu, for example, as high as 5-10 ppb (μg/ℓ) (Erickson et al., 1970; J.H. Gentile, personal communication*). Unwanted trace metals can be removed by passing the sea water through a column containing a deionizing resin (Davey et al., 1970), but this method may not be practical for large volumes of water.

Many effluents to be tested are complex mixtures having both solid and liquid components. There may also be gaseous components. The following guidelines should be followed when preparing test media:

---

*J.H. Gentile, National Marine Water Quality Laboratory, South Ferry Road, Narragansett, Rhode Island 02882

If Liquid Only--

Waste material should be stirred or shaken thoroughly before use. Waste material may be used directly or as in a stock prepared by dilution with filtered sea water to a desired concentration. All stocks and test concentrations should be prepared on a weight-to-volume basis (gm/ℓ, mg/ℓ, µg/ℓ). If volume/volume basis is used, a correction should be made for specific gravity of the material being tested, i.e., (weight/volume) (specific gravity) = volume/volume.

If Solid and Liquid--

It may be desirable to test the effects of solids on Palaemonetes larvae. Solids can be added to the test containers by weight and agitated to keep them in suspension; combined toxic-mechanical effects then are determined. Alternately, one volume of solid material may be diluted with four volumes of sea water to prepare a standard elutriate.

All test glassware should be thoroughly washed, using the following procedure:

1) Empty old test solution and rinse with cold water.

2) Rinse with acetone, followed by a warm water rinse.

3) Wash with laboratory soap and a brush. Rinse thoroughly with warm water 4-5 times.

4) Rinse with 10% HCl or HCO_3, if the toxicant contains heavy metals.

5) Rinse with distilled water and allow to dry. If an acid rinse is used, it should be followed by 4-5 thorough rinses with deionized water.

Bioassay Procedures

Because Palaemonetes normally exhibits variability in molting and developmental rates during larval life, it is not feasible to produce sufficient larvae of individual stages for testing. Therefore, tests use larvae of specified ages (e.g., ages 1 and 18 days). Most larvae will metamorphose to postlarvae (PL) on approximately day 18-21. Hence, one bioassay is performed on 18-day-old larvae, and one on postlarvae, to determine if the biochemical and physiological changes accompanying metamorphosis alter the response to the toxicant. For the same reasons, a bioassay using day 30 postlarvae may be required.

Palaemonetes larvae are added to test containers, using a method of random selection (total randomization, stratified randomization, etc.). Larvae are removed from culture dishes, using a rectangular piece of fine mesh Nitex nylon net, and stocked in test dishes at a density of 30 larvae/liter of test media/culture dish. Larvae are fed an excess supply of Artemia nauplii throughout the test. Artemia are added with each change of test media. Mortalities of Palaemonetes are recorded at the time of each test media change (every 12 or 24 hours), and all dead animals removed at this time. All test and control culture dishes should be maintained at 25°C in a culture cabinet, BOD incubator, or water table. Tests may be run in total darkness or on a 12-hr light - 12 hr-dark regime. All tests should include 4-6 concentrations and a sea water control. Control mortality exceeding 10% invalidates test results. Because of the inherent variability of each age group of larvae, 2 to 3 replicates must be run simultaneously for each test concentration in each experiment. These basic test conditions are to be followed for both range-finding and definitive bioassays, as discussed below. (See Figures 1-I and 2-I.)

Initially, a series of range-finding 96-hour assays are performed, using 1- and 18-day-old larvae to determine the range of toxicity of the material being examined, and to determine the best test conditions. A broad range of concentrations covering at least four orders of magnitude should be tested; e.g., 0.01, 0.1, 1.0, and 10 mg/ℓ (ppm), or gm/ℓ (%). Temperature, pH, and dissolved oxygen (DO) levels should be monitored throughout these tests to help determine need for aeration and frequency of test solution change.

After the range-finding test is completed, a LC50, concentration lethal to 50% of the shrimp, is approximated and a series of definitive bioassays performed. The purpose of the definitive bioassay is to more clearly determine the limits of toxicity of a waste and better estimate the LC50. Concentrations chosen for definitive bioassays are determined by results from the range-finding tests, i.e., the lowest definitive test concentration should equal or be greater than the greatest concentration in range-finding tests that killed few or no test organisms. Likewise, the greatest definitive test concentration should be equal to or less than the least concentration in range-finding tests that killed all or almost all test organisms. (See Figures 1-I and 2-I.) Once upper and lower definitive test concentrations are chosen, intermediate concentrations are calculated, using progressive bisection of intervals on a logarithmic scale (Standard Methods, 1965). At least five, and preferably more, test concentrations are used to yield mortality data on either side of a 50% kill, a condition necessary for statistical treatment of data using Probit Analysis.

Growth is often a more sensitive indication of effect than mortality and is useful in choosing concentrations to be used for chronic tests. Therefore, at the end of each test, the rostrum-telson length of surviving larvae from each test concentration and controls should be measured with an ocular micrometer. A sample of untreated larvae should be measured at the beginning of the test for comparative purposes. Additional observations, such as loss of equilibrium, cessation of feeding, irregular movements, and other behavioral aberrations, should be noted at the time of each test media change.

Analysis of Data

Data from 96-hour acute bioassays should be analyzed by Probit Analysis (Finney, 1964a, b). This method estimates a value for LC30, 70, and 90, as well as the LC50. Because Probit Analysis is generally performed by computer, it is wise to check the computer output by plotting percentage kill in probits against logarithm of concentration, and comparing computer and graphed LC50s. The line thus plotted should closely resemble that determined by the computer. The Litchfield-Wilcoxon method of LC50 estimation or graphical interpolation, using Probit graph paper (Standard Methods, 1965), can be used when data do not meet the more rigorous specifications required by Probit Analysis (Litchfield, 1949; Litchfield and Wilcoxon, 1949, 1953). The 95% confidence limits should be indicated for all data.

Reports

At the completion of testing and data analysis, a report is usually required. Such reports should include the following information:

1. Name of method, investigator, laboratory, and date tests were conducted.

2. Detailed description of material tested, source, date, and time of collection, composition, known physical and chemical properties.

3. Source of sea water, date, and method or preparation.

4. Detailed information about test animals, including scientific name, life stage, age, source, history, and acclimation procedure for larvae, if appropriate.

5. Experimental design, test containers, volume of test solution, initial test conditions, number of organisms at each concentration, number of organisms in each control, and types of controls run.

6. Definitions of response used to determine the effect under investigation and a summary of general observations of related effects or symptoms.

7. Percentage of control organisms that died or were affected during the test.

8. LC50, with confidence limits. LC30, 70, and 90, if pertinent.

9. Methods used for and results of all DO, pH, and temperature measurements.

10. Any deviations and reasons.

11. Other relevant information.

REFERENCES

Broad, A. Carter. 1955. Reproduction, Larval Development and Metamorphosis of Some Natantia from Beaufort, N.C. Ph.D. Thesis. Duke University. 87 pp.

____. 1957a. Larval Development of Palaemonetes pugio Holthuis. Biol. Bull. 112:144-161.

____. 1957b. The Relationship Between Diet and Larval Development of Palaemonetes. Biol. Bull. 112:162-170.

____ and Jerry H. Hubschman. 1962. A Comparison of Larvae and Larval Development of Species of Eastern U.S. Palaemonetes With Special Reference to the Development of Palaemonetes intermedius Holthuis. Am. Zool. 2(3):172 (Abstr.).

Davey, E.W., J.H. Gentile, S.J. Erickson and P. Betzer. 1970. Removal of Trace Metals from Marine Culture Medium. Limnol. Oceanog. 15(3):333-490.

Erickson, S.J., N. Lackie and T.E. Maloney. 1970. A Screening Technique for Estimating Copper Toxicity to Estuarine Phytoplankton. J. Water Pollut. Control Fed. 42:R270-R278.

Federal Register, Part II. 1973. U.S. Environmental Protection Agency--Ocean Dumping Criteria, May 16, 1973. 38(94):12874.

Finney, D.J. 1964a. Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve. Cambridge at the University Press, Cambridge. 318 pp.

____. 1964b. Statistical Method in Biological Assay. 2nd Ed. Hafner, N.Y. 668 pp.

Holthuis, L.B. 1949. Notes on the Species of Palaemonetes (Crustacea, Decapoda) Found in the United States of America. K. Ned. Akad. v. Wet. 52:87-95.

____. 1952. A General Revision of the Palaemonidae (Crustacea, Decapoda, Natantia) of the Americas. II. The Subfamily of Palaemoninae. Occas. Pap. Allen Hancock Found. 12:1-369.

Hubschman, J.H. and A.C. Broad. 1974. The Larval Development of _Palaemonetes intermedius_ Holthuis, 1949 (Decapoda, Palaemonidae) Reared in the Laboratory. Crustaceana 26(1):89-103.

Litchfield, J.T., Jr. 1949. A Method For Rapid Graphic Solution of Time-percent Effect Curves. J. Pharmacol. Exp. Ther. 97:399-408.

____ and F. Wilcoxon. 1949. A Simplified Method of Evaluating Dose-effect Experiments. J. Pharmacol. Exp. Ther. 96:99-113.

____. 1953. The Reliability of Graphic Estimates of Relative Potency from Dose-percent Effect Curves. J. Pharmacol. Exp. Ther. 108:18-25.

Little, Georgiandra. 1968. Induced Winter Breeding and Larval Development in the Shrimp, _Palaemonetes pugio_ Holthuis (Caridea, Palaemonidae). Crustaceana, Supplement 2: Studies on Decapod Larval Development. 19-26 pp.

Sandifer, Paul A. 1973. Effects of Temperature and Salinity on Larval Development of Grass Shrimp, _Palaemonetes vulgaris_ (Decapoda, Caridea). U.S. Fish. Wildlf. Serv. Fish Bull. 71(1):115-123.

Standard Methods for the Examination of Water and Wastewater. 12th Ed. 1965. American Public Health Association, Inc. New York, N.Y. 769 pp.

J. ENTIRE LIFE-CYCLE TOXICITY TEST USING GRASS SHRIMP (*PALAEMONETES PUGIO* HOLTHUIS)

Dana Beth Tyler-Schroeder

INTRODUCTION

The purpose of this method is to assess toxicity of a material to all life stages of the grass shrimp in flow-through systems. This experiment determines effects on survival, growth, and reproduction (including number of females spawning, number of days before onset of spawning, number of eggs per female, and hatching success) of parental generation shrimp. Effects on survival, larval development, and growth are also determined for F_1 generation shrimp. These tests must extend through an entire life-cycle of the shrimp--from juvenile stage of the parental generation, sexual maturation and reproduction, through hatching, larval development, and growth of the F_1 generation to juvenile stage. Tests may terminate at this point, or exposures can be continued if necessary to determine effect on F_1 reproduction and F_2 larval development.

Basic methodology for flow-through toxicity tests has been described in the section, "Entire Life-Cycle Toxicity Test Using Sheepshead Minnows (*Cyprinodon variegatus*)" (Hansen et al., this publication) and by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). The following procedures describe only aspects unique to toxicity testing with the grass shrimp.

PHYSICAL SYSTEMS

Salinity of test water should be 20 $^{o}/oo$, to be near the salinity optima for larval development (25 $^{o}/oo$) (Floyd, 1977). Test water temperatures are controlled to 25°C ± 1°C. Photoperiod regimes for life-cycle toxicity tests vary and are discussed here and in the section describing laboratory cultivation of grass shrimp.

A Mount and Brungs diluter (1967) with appropriate modifications has been successfully employed in life-cycle toxicity tests. The diluter should deliver one-half liter to each exposure aquaria for each cycle. If acetone, triethylene glycol, ethanol, or other solvents are used as carriers, the diluter must be modified to provide equal carrier concentrations in all exposure water. A carrier control and a control without carrier must be provided (Schimmel et al., 1974).

Exposure aquaria, 1/4-inch plate or 1/8-inch double strength glass, are constructed with a clear, silicone rubber sealant. All materials in exposure aquaria or equipment must be glass, or another inert material, such as nylon, teflon, etc.

Test aquaria of 40 cm x 61 cm x 22 cm deep with a water depth of 17 cm were successfully utilized in a life-cycle toxicity test with grass shrimp exposed to the chlorinated hydrocarbon pesticide, endrin (Tyler-Schroeder, in press). The drain is constructed to resemble that in the tank used to capture larvae. (See "Culture of Grass Shrimp (Palaemonetes pugio) in the Laboratory.") The plexiglas disc is of approximately 5-cm diameter, and the drain pipe must bend at a 90° angle to drain through the front rather, than the bottom of the tank. The nylon screen collar is constructed of 363 μm mesh and must extend one to two centimeters above water level.

BIOLOGICAL ASPECTS

Grass shrimp for toxicity testing may be obtained from natural or laboratory populations. Test animals must be uncontaminated, i.e., whole body residue analyses must be free of unacceptable concentrations of pesticides, PCB's, heavy metals, or other pollutants of concern.

Juvenile grass shrimp are acclimated in the laboratory under conditions described for the life-cycle toxicity test. Mortality can be no greater than two percent during the 4-day acclimation period. Near darkness (15-watt bulb turned on only for feeding, daily cleaning, and observations) is maintained during acclimation to inhibit premature reproduction.

Adult and juvenile shrimp are fed fish flakes (such as Metaframe^R HiProMin Tropical Flakes or TetraMin^R) that contain both plant and animal material during acclimation and testing. A supplement of frozen adult brine shrimp or newly hatched brine shrimp nauplii is added during growth until sexual maturity and induction of spawning. Grass shrimp larvae must be fed newly hatched brine shrimp nauplii several times a day. At the time of metamorphosis to postlarvae, the diet may be changed to the fish flake. Foods must not be contaminated with pesticides, PCB's, heavy metals, or other pollutants of concern.

TEST REGIME

Initial Stage

Start the life-cycle toxicity test with 100 juvenile shrimp (less than 15 mm rostrum-telson length) randomly distributed in each test concentration. Gonad development must not be obvious at the start of the test. Exposure aquaria are examined daily to count and remove dead shrimp. Daily counts of surviving shrimp are impractical; however, individual shrimp are to be counted every four to six weeks. Lengths of 30 shrimp per concentration are measured at the start of the test and every four weeks until termination to determine effects on growth. All shrimp lengths discussed here refer to rostrum-telson length. (The shrimp is extended to its full length and measured from the tip of the rostrum to the end of the uropods of the telson).

During the time required for pollutant uptake and growth of shrimp to sexual maturity (usually two to three weeks), the photoperiod is held constant at 8L:16D, using 15-watt, 125-lumen incandescent bulbs to prevent premature induction of gonad development and spawning. The length of this initial exposure is based on the time necessary for the pollutant to reach equilibrium between shrimp tissue and the concentration in water. Preliminary bioconcentration exposures are conducted according to methods prescribed by Hamelink (1977).

Induction of Spawning

After approximately a two- to three-week exposure, shrimp should average 20 to 25 mm in length. Spawning is induced, using 100-watt, 1750-lumen incandescent lamps and 10L:14D photoperiod. Thereafter, photoperiod is increased by 47-minute increments every two weeks to a maximum 15-hr, 29-min light:8-hr, 31-min darkness.

When production of ovigerous females is first noticed, a partition of 2.0 mm nylon mesh is installed in the tank at a distance of 11 cm from the front. Ovigerous females are separated from the rest of the population to allow an accurate count of number of females spawning per day.

Effects on Reproductive Success

Number of ovigerous females produced in each exposure concentration must be recorded daily. Eggs from at least 10 females per concentration must be counted. Rostrum-telson length of each female is recorded with the respective egg count because egg production is proportional to length. This recorded length must be used as a covariate in statistical analyses.

Effects on hatching success of embryos is determined in a hatching apparatus and larval rearing tray that are installed in each aquarium once females are ovigerous and eggs have darkened. Each hatching apparatus consists of a 6.35-mm, I.D. tubing manifold that delivers water to five glass spawning chambers. A spawning chamber is constructed of a 30-mm diameter glass tube, 8.5-mm long fitted with neoprene stoppers and input and output tubing on either end (Tyler-Schroeder, in press). One ovigerous female is placed in each spawning chamber to facilitate individual egg counts. Water from the diluter flushes newly hatched larvae from each chamber into an egg cup constructed of a 100-mm diameter Petri dish top fitted with a nylon mesh collar (363μm) 13 mm high. Larvae hatched from at least 10 females in each exposure concentration are counted and compared to egg counts to estimate hatching success.

The egg cups are held in larval hatching and rearing trays placed in each aquarium during reproductive and larval development phases of the test.

Each tray consists of an elevated tank supported by rectangular glass legs on two ends; the glass legs form each end of the tank. Hatching trays, 34 cm x 43 cm x 13 cm high, are constructed of 1/8-inch double-strength glass. Water depth is controlled by a self-starting siphon, causing the water level to fluctuate from 8 to 11.5 cm. Thus, water from the diluter flows in and out of egg cups.

Larval Development and Metamorphosis

After recording hatching success, the hatching apparatus is removed from the larval-rearing tray. Larvae hatched from several females are placed in larger diameter egg cups (150-mm Petri dish tops, with 363 μm nylon mesh collar 15 mm high) to observe effects on larval development (20 larvae/egg cup; four to five egg cups per concentration). Larvae are counted daily to record effects on survival, length of larval development, and metamorphosis. Larvae metamorphose to postlarvae within 12 to 20 days of hatching.

Effects on Growth

Thirty-five days after hatching, the rostrum-telson length of all postlarvae are measured to determine pollutant effects on growth. Postlarvae are subsequently released from the egg cups into the larval-rearing tray. The self-starting siphon is replaced with a screened drain to prevent escape of small shrimp from the hatching and rearing tray. Lengths of at least 30 shrimp per concentration are recorded at weekly intervals to evaluate effect on growth.

Additional Tests

In order to develop application factors used in setting water quality criteria, it is necessary to determine "effect" and "no-effect" concentrations in a life-cycle toxicity test and compare these to the 96-hr LC50 for juvenile shrimp determined in an acute toxicity test (Eaton, 1973). Methods for acute toxicity tests in flow-through systems have been described by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975) and by other authors in this manual. These methods should be consulted and a 96-hour LC50 obtained.

Test Termination

Termination of a life-cycle toxicity test depends upon nature of the pollutant being tested, its intended use and disposal pattern, and the kind of information desired. Technically, if the test began with 15-mm juveniles, a full life-cycle has been completed by the time F_1 larvae metamorphose to postlarvae and grow to 15 mm long juveniles. Some chemicals are particularly persistent in the environment or are released into the environment in consistent amounts over a long period of time. When testing such pollutants, it may be desirable to continue the life-cycle toxicity test exposures until effects on F_1 reproduction and F_2 hatching success, larval development, and growth can be assessed. In either case, the test is terminated after shrimp of the youngest desired generation (F_1 and F_2) have completed larval development, metamorphosed to postlarvae, and grown to juveniles approximately 15 mm long.

At test termination, all surviving shrimp (parental generation shrimp that spawned, parental generation shrimp that did not spawn, F_1 generation shrimp) are individually measured (rostrum-telson length). Shrimp from each test concentration are grouped as above (parental shrimp that spawned, parental shrimp that did not spawn, F_1 generation shrimp) and a composite weight recorded for each grouping. The number of shrimp in each group is recorded to calculate individual weight per shrimp in each group or to calculate average weight of individual shrimp in each test concentration. Residue analyses of whole body shrimp are made with the same groupings per test concentration as above. Shrimp are quickly killed by brief exposure to a stream of steaming hot water.

A final count of surviving shrimp is made for each test concentration (using the same groupings as above) to determine effects on survival.

STATISTICAL ANALYSES

Data from the life-cycle toxicity test are analyzed by analysis of variance and multiple comparison methods to determine differences between means. All data compared as percentages (e.g., percent survival, percent metamorphosis, etc.) should be transformed, using the arc sine transformation (Winer, 1971).

The data related to effects on egg production and larval hatching are compared by analysis of covariance, the covariate being the rostrum-telson length of the female from which eggs or hatched larvae were produced. Appropriate multiple comparison methods are used to determine differences among means.

The data from the juvenile 96-hour acute toxicity test are analyzed by linear regression after probit transformation to determine LC50's and 95 percent confidence limits. Uptake and depuration rates from bioconcentration studies are calculated by the nonlinear statistical model of Bahner and Oglesby (in press).

REFERENCES

Bahner, L.H., and J.L. Oglesby. (In Press). Test of Model for Predicting Kepone Accumulation in Selected Estuarine Species. American Society for Testing and Materials.

Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. EPA Report No. EPA-660/3-75-009. U.S. Environmental Protection Agency, Washington, DC. pp. 61.

Eaton, J.G. 1973. Recent Developments in the Use of Laboratory Bioassays to Determine "Safe" Levels of Toxicant. G.E. Glass (ed.) Ann Arbor Science Publishers, Inc., Ann Arbor, MI. 107-115.

Floyd, W.R. 1977. The Effects of Temperature and Salinity on the Larval Development of the Grass Shrimp, Palaemonetes pugio Holthuis, Reared in the Laboratory. Master's Thesis, Old Dominion University, Dept. of Oceanography, Norfolk, VA. 145 pp.

Hamelink, J.L. 1977. Current Bioconcentration Test Methods and Theory in Aquatic Toxicology and Hazard Evaluation, ASTM STP 634, F.L. Mayer and J.L. Hamelink, eds., American Society for Testing and Materials, pp. 149-161.

Mount, Donald I., and William Brungs. 1967. A Simplified Dosing Apparatus for Fish Toxicology Studies. Water Res. 2: 21-29.

Schimmel, Steven C., David J. Hansen, and Jerrold Forester. 1974. Effects of Aroclor^R 1254 on Laboratory-Reared Embryos and Fry of Sheepshead Minnows (Cyprinodon variegatus). Trans. Am. Fish. Soc. 103(3): 582-586.

Tyler-Schroeder, D.B. (In Press). Use of the Grass Shrimp, Palaemonetes pugio, in a Life-Cycle Toxicity Test. American Society for Testing and Materials.

Winer, B.J. 1971. Statistical Principles in Experimental Design. McGraw-Hill Book Company. New York, NY. 907 pp.

K. STATIC METHOD FOR ACUTE TOXICITY TESTS USING FISH AND MACROINVERTEBRATES

(See list of contributors.)

EQUIPMENT

Facilities

For maximum convenience and versatility, the facilities should include tanks or cages to hold and acclimate test animals, a tank for salt water, a temperature-controlled recirculating water bath, or an environmentally controlled room for the test containers. The holding and acclimation tanks should be equipped for temperature control, and the holding tank should be equipped for aeration. Because air used for aeration must not contain oil or fumes, it must be taken from a well-ventilated, fume-free area, and powered by a surface aerator or an oil-free rotary or piston-type air compressor. During holding, acclimation, and testing, test animals should be shielded from disturbances.

Construction Materials

Construction materials and commercial equipment that might contact water in which test animals will be placed should not contain any substances that can be leached or dissolved by the water. In addition, materials and equipment should be chosen to minimize sorption of toxicants from water. It is suggested that glass, #316 stainless steel, or perfluorocarbon plastics be used when possible.

Test Containers

1. Type: The test solution for fish and invertebrates should be placed in containers measuring between 15 and 20 centimeters (cm) deep. These animals can be tested in 19.6ℓ (5-gallon) wide-mouthed soft glass bottles containing 15ℓ of solution. Alternatively, test containers can be made by welding (not soldering) stainless steel or by gluing double-strength window glass with clear silicone adhesive. As little adhesive as possible should be in contact with the water; extra beads of adhesive should be on the outside of the containers rather than on the inside. Some invertebrates can be tested in 3.9ℓ (1-gallon) wide-mouthed soft glass bottles or battery jars containing 3ℓ of solution.

2. Cleaning: Test containers must be cleaned before use. New containers must be washed with detergent and rinsed with 10% hydrochloric acid, acetone, and tap or other clean water. The containers, if reused after a test, should be (1) emptied; (2) rinsed with water; (3) cleaned by an appropriate procedure to remove the test toxicant, e.g., acid to remove metals and bases; detergent, organic solvent, or activated charcoal to

remove organic compounds; and (4) rinsed with water. Acid is useful for removing scale, and hypochlorite (bleach) is useful in removing organic matter and as a disinfectant. All test containers must be rinsed with salt water immediately before use.

Salt Water

Acute toxicity tests require salt water in which healthy animals can survive throughout acclimation and testing without sign of stress, such as unusual behavior or discoloration. Salt water is prepared from commercially available formulations or from ingredients listed in Table 1-K, using deionized or glass-distilled water. Deionized or distilled water is used to dilute the salt water to a salinity of 30 parts per thousand (^{o}/oo). Natural salt water that satisfies the acclimation requirement also can be used.

TABLE 1-K. STANDARD SALT WATER*

Ingredient	Amount (g)	Ingredient	Amount (g)
$SrCl_2.6H_2O$	0.02	$MgCl_2.6H_2O$	10.0
H_3BO_3	0.03	NaCl	23.50
KBr	0.10	$Na_2SiO_3.9H_2O$	0.02
$CaCl_2.2H_2O$	1.10	EDTA†	0.003
Na_2SO_4	4.00		

*To formulate this water, mix technical grade salts with 900 mℓ of distilled or demineralized water in the order and quantities listed. Then add enough distilled or demineralized water to make the final volume 1ℓ. Dilute the water with distilled or demineralized water to achieve a salinity of 30 ^{o}/oo. If necessary, add NaHCO to adjust final pH of water to between 8.0 and 8.2. Before use, filter water through a 0.22-micron membrane filter.

†Ethylenediaminetetracetate.

Test Organisms

Species--

The Regional Administrator shall designate the appropriate test animals to be used in a particular region. Test animals are as follows (specific names must be reported):

Invertebrates:

White sea urchin, *Tripneustes esculentus*
White shrimp, *Penaeus setiferus*
Pink shrimp, *P. duorarum*
Brown shrimp, *P. aztecus*
Grass shrimp, *Palaemonetes* sp.
Shrimp, *Crangon* sp.
Oceanic shrimp, *Pandalus jordani*
Blue crab, *Callinectes sapidus*
Dungeness crab, *Cancer magister*

Vertebrates:

Sheepshead minnow, *Cyprinodon variegatus*
Mummichog, *Fundulus heteroclitus*
Silverside, *Menidia* sp.
Threespine stickleback, *Gasterosteus aculeatus*
Pinfish, *Lagodon rhomboides*
Spot, *Leiostomus xanthurus*
Shiner perch, *Cymatogaster aggregata*
Buffalo sculpin, *Enophrys bison*
Pacific staghorn sculpin, *Leptocottus armatus*
English sole, *Parophrys vetulus*

Other species indigenous to the dumping area can be used and are preferred, if approved by EPA. The specific name of the animals must be verified and reported. Samples of the test animals can be requested by EPA. Tests on other animals under other experimental conditions may be required by EPA.

Source--

Test animals are usually collected from wild populations in relatively unpolluted areas. (Collecting permits may be required by local or state agencies.) Some animals can be purchased from commercial suppliers or reared in the laboratory. (See culture techniques.) All animals should be healthy and as uniform in size and age as possible.

Size--

1. Fish: Fish that weigh between 0.5 and 5.0 grams each are usually desirable. In any single test, the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than two times the standard length of the shortest fish.

2. Maximum size of invertebrates:

shrimp--less than 10-cm rostrum-telson length,
crabs--less than 10-cm carapace width.

Since cannibalism occurs in many species, the claws of crabs should be banded, or the individuals should be physically isolated.

Care and handling--

If the animals are to be tested at a temperature or salinity other than that at which they are collected, they should not be subjected to more than a 3°C change in water temperature in any 24-hour period or to more than a 5 °/oo change in salinity in any 24-hour period. Crowding should be avoided to maintain animals in good condition during holding and acclimation. Animals should be fed at least once a day if held for an extended period, and tanks should be cleaned after feeding.

Animals should be handled as little as possible. Any necessary handling should be done as gently, carefully, and quickly as possible.

Test animals cannot be taken from any group of organisms apparently diseased or otherwise stressed, or from any group in which more than 3 percent of the individuals died during the 48 hours immediately prior to establishing test containers.

Recommended Procedure for Testing Material

Experimental Design--

At least 10 control and 10 test animals must be exposed to each concentration or dilution of the test material. (They can be in two or more containers.) However, use of additional animals and replication of treatments are desirable. Replicates, if used, should have no water connection between replicate test containers. Exposures can be conducted by stratified randomization (random assignment of one test container for each treatment in a row, followed by random assignment of a second test container for each treatment in another or extended row) or by total randomization. A representative sample of test animals should be distributed impartially to test containers by adding one animal (when less than 11 are used) or two animals (when more than 11 are used), and then repeating this process until desired number of test animals is reached in each container. Animals can be assigned alternatively either by total randomization or by stratified randomization (random assignment of one animal to each test container, random assignment of a second animal to each test container, etc.).

Controls for every test must duplicate the salt water conditions, and animals used in containers with test material. A test is not acceptable if more than 10 percent of the control animals die.

Temperature--

Test water temperature must be maintained within 1°C of the water temperature listed in Table 2-K.

TABLE 2-K. SUGGESTED SEA WATER TEST TEMPERATURES FOR VERTEBRATES AND INVERTEBRATES*

Region	Temperature
I	20°C
II† and III	25°C
IV, VI and IX	30°C
X	15°C

*Temperatures in this table should be revised to the highest average monthly temperature of oceanic surface waters at dump sites in each region.

†Puerto Rico and Virgin Islands are in Region II administratively but should use temperatures suggested for Region IV.

Salinity test--

Test water salinity should be 30 °/oo before the material to be tested is added.

Loading--

The mass of animals in each test container must be limited so that the animal's oxygen requirements do not influence the test results. In general, test containers should not contain more than one gram of animals per liter. Tests at high temperature may require reduced loading. Proper loading can be confirmed by measuring dissolved oxygen concentration in the water of the unaerated control containers. It must not be less than 40% saturation.

Preparation of Material to be Tested (See other section of manual on this subject)--

Samples, whether liquid waste or sludge, must be stirred to a uniform consistency before dilutions are made.

Concentrations--

Dilutions of samples, by volume, of 10% (100,000 ppm, 100 ml/ℓ) 1% (10,000 ppm, 10 ml/ℓ), 0.1% (1,000 ppm, 1 ml/ℓ), 0.01% (100 ppm, 0.1 ml/ℓ), 0.001% (10 ppm, .01 ml/ℓ), and 0.0001% (1 ppm, 0.001 ml/ℓ) are recommended as initial test concentrations. In some instances, concentrations of >10% must be tested and resultant salinity adjusted to that of control.

The highest concentration (dilution) is prepared as follows: 9 volumes of salt water are added to 1 volume of the stirred sample. (Adequate space should be reserved in the test container for stirring and addition of animals.)

Each succeeding concentration is prepared by a similar 1-in-10 serial dilution from the previous test container. Adequate stirring of the contents of the test container is essential before each dilution.

Transfer of Animals--

Animals must be added to the test containers within 1 hour after the proper dilutions of the material to be tested have been made.

Feeding--

The organisms must not be fed while in the test containers except for Mysidopsis, and these organisms must be fed daily. (See pages 59 and 62.)

Measurements--

The dissolved oxygen concentration, pH, and temperature must be measured (1) before adding animals and (2) at 24-hour intervals thereafter in the highest and lowest concentration and in the control. Additional measurements are required in containers in which animals die. Water samples should be taken midway between the top, bottom, and sides of the test containers and should not include any surface scum of material stirred up from the bottom or sides.

Observations--

At a minimum, the number of dead or affected animals must be recorded at 24-hour intervals throughout the test. More observations are often desirable, especially near the beginning of the test. Dead animals must be removed as soon as they are observed

The adverse effect most often used to study acute toxicity with aquatic animals is death. Criteria for death are no movements, especially no opercular movement in fish, and no reaction to gentle prodding. However, death is not easily determined for some invertebrates; thus an EC50 (effective concentration to 50% of test animals) is often measured rather than an LC50 (lethal concentration to 50% of test animals). The effect generally used for determining an EC50 with invertebrates is immobilization, which is defined as the inability to move, except for minor activity of appendages, or loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucous production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism should be reported.

Calculation and Reporting

At the end of the test period, the bioassays are terminated and the LC50 or EC50 values are determined.

Calculations--

An LC50 is a concentration at which 50% of the experimental animals died, and an EC50 is a concentration at which 50% of the experimental animals were affected. Either may be an interpolated value based on percentages of animals dying or affected at two or more concentrations. Estimating the LC50 or EC50 by interpolation involves plotting the data on semilogarithmic coordinate paper with concentrations on the logarithmic axis and percentages of dead or affected animals on the arithmetic axis. A straight line is drawn between two points representing death or effect in concentrations that were lethal to or effective against more than half and less than half of the organisms. The concentration at which the line crosses the 50% mortality or effect line is the LC50 or EC50 value. If 50% of the test animals are not affected by the highest concentration, the percentage affected should be reported.

Reports--

Any deviation from this method must be noted in all reports of results. A report of the results of both aerated and unaerated tests must include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source, date and time of collection, composition, known physical and chemical properties, and variability;

3. the source of the salt water, date prepared, and method of preparation;

4. detailed information about the test animals, including name, standard length, weight, source, history, and acclimation procedure used;

5. a description of the experimental design, the test containers, the volume of test solution, initial test conditions, the number of organisms per concentration, and the loading;

6. definitions of the criteria used to determine the effect and a summary of general observations on other effects or symptoms;

7. percentage of control organisms that died or were affected in each test container;

8. the 24-, 48-, and 96-hour LC50, or EC50;

9. methods used for and the results of all dissolved oxygen, pH and temperature measurements; and

10. any other relevant information.

L. FLOW-THROUGH METHODS FOR ACUTE TOXICITY TESTS USING FISHES AND MACROINVERTEBRATES (See list of contributors.)

INTRODUCTION

Continuous-flow (often referred to as "flow-through") bioassays have definite advantages over static tests in evaluating certain types of wastes to be disposed of at sea, particularly in testing waste chemicals that have high biochemical oxygen demands, and are unstable or volatile. Many test species of fish and macroinvertebrates have high rates of metabolism and are difficult to maintain in jars or tanks of standing sea water. Continuous-flow bioassays, conducted under proper conditions, provide for well-oxygenated test solutions, nonfluctuating concentrations of the toxicant, and continual removal of metabolic wastes of the test organisms (Standard Methods, 13th Edition, 1971).

This method provides general procedures for conducting a 96-hour, flow-through bioassay on marine fish and macroinvertebrates such as shrimp and crabs. Evaluation of different types of waste will require some modification of these procedures.

Equipment

Facilities--

For maximum convenience and versatility, the facilities should include tanks or aquaria for holding and acclimating test animals, a tank for sea water, and a temperature-controlled recirculating water bath or controlled-environment room for the test containers. The holding and acclimation tanks should be equipped for temperature control and the holding tank should be equipped for aeration for emergency use. During holding, acclimation, and testing, test animals should be shielded from unnecessary disturbances.

Construction Materials--

Construction materials and commercially purchased equipment that may contact any water into which test animals are to be placed should not contain any toxic substances that can be leached, corroded, or dissolved by the water. In addition, materials and equipment should be chosen to minimize sorption of toxicants from water. It is suggested that glass, #316 stainless steel, or perfluorocarbon plastics be used whenever possible.

Test Containers--

Type: The test solution for fish and invertebrates usually should be placed in containers measuring between 15 and 20 cm deep. Test containers can be made by welding (not soldering) stainless steel or by gluing double-strength window glass with clear silicon adhesive. As little adhesive as possible should be in contact with the water; extra beads of adhesive should be on the outside rather than inside the containers. Plywood tanks coated with fiberglass resin are also acceptable.

Cleaning: Test containers must be cleaned before use. New containers must be washed with detergent and rinsed with 10% hydrochloric acid, acetone, and tap or other clean water. Test containers, if reused, should be (1) emptied; (2) rinsed with water; (3) cleaned by an appropriate procedure to remove the toxicant tested, e.g., acid to remove metals and bases; detergent, organic solvent, or activated charcoal to remove organic compounds; and (4) rinsed with water. Acid is also useful for removing scale and hypochlorite (bleach) is useful for removing organic matter and for disinfecting. All test containers must be rinsed with uncontaminated sea water immediately before use.

Sea Water

Acute toxicity tests, require acceptable sea water in which healthy test animals can survive throughout acclimation and testing without sign of stress, such as unusual behavior or discoloration. Natural sea water (particularly from the dump site) is preferable to artificial sea water; however, artificial sea water is sometimes more practical due to logistics or costs. Salinity of test water ideally should duplicate the dump site; however, requirements of the individual species to be tested must be considered. See page 21 for composition of artificial sea water.

Test Organisms

Species--

Recommended species are as follows (specific name must be verified and reported):

Invertebrates:

White sea urchin, *Tripneustes esculentus*
White shrimp, *Penaeus setiferus*
Pink shrimp, *P. duorarum*
Brown shrimp, *P. aztecus*
Grass shrimp, *Palaemonetes* sp.
Shrimp, *Crangon* sp.
Oceanic shrimp, *Pandalus jordani*
Blue crab, *Callinectes sapidus*
Dungeness crab, *Cancer magister*

Vertebrates:

Sheepshead minnow, Cyprinodon variegatus
Mummichog, Fundulus heteroclitus
Longnose killifish, F. similis
Silverside, Menidia sp.
Threespine stickleback, Gasterosteus aculeatus
Pinfish, Lagodon rhomboides
Spot, Leiostomus xanthurus
Shiner perch, Cymatogaster aggregata
Buffalo sculpin, Enophrys bison
Pacific staghorn sculpin, Leptocottus armatus
English sole, Parophrys vetulus

Other species indigenous to the dumping area can be used if approved by EPA and if the specific name of the organism is verified and reported. Samples of the test animals can be requested by EPA. Tests on other organisms under other experimental conditions can be required by EPA.

Source--

Test animals are usually collected from wild populations in relatively unpolluted areas. (Collecting permits may be required by local or state agencies.) Some animals can be purchased from commercial suppliers. All animals should be healthy and as uniform in size and age as possible. Juvenile stages are preferable.

Size--

1. Fish: Fish that weigh between 0.5 and 5.0 g each are usually desirable. In any single test, the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than two times the standard length of the shortest fish.

2. Size requirements for invertebrates:
Palaemonetes--10-20 mm rostrum-telson length
shrimp--5-8 cm rostrum-telson length (5-8 g live weight)
crabs--less than 7 cm carapace width

Acclimation--

Conditions of acclimation should be related to test requirements. Organisms should be subjected to as little stress as possible. Initially, temperature and salinity in the laboratory should resemble those of the medium used to transport test animals. During acclimation, mortality should not exceed 10 percent. Fishes should be held in the laboratory at least 14 days and invertebrates 4 days prior to testing. If the acclimation temperature and salinity differ from those of the test, they should be adjusted gradually (at least 48 hours prior to testing) to the test conditions.

Care and Handling----

Animals should be handled as little as possible. Any necessary handling should be done with a dip net as gently, carefully, and quickly as possible. Animals should be fed daily during acclimation, but fish should not be fed for a period of 48 hours before or during the actual test. It may be necessary, however, to feed certain invertebrates during the actual test.

Crowding should be avoided. Cannibalism occurs in many species of arthropods; therefore, in some cases it may be necessary to isolate individuals in compartmented aquaria by such techniques as banding the crab claws, and placing a 2-3 cm (about 1 in) layer of sand in the bottom of the aquaria used for testing shrimp.

Recommended Procedure for Testing Materials

Experimental Design----

The recommended test procedure consists of a 96-hour bioassay, using a control and at least five concentrations of the material to be tested. Acute static tests are useful in determining range of toxicity of the material and selecting concentrations for the flow-through tests. (See Section 5-f, Range-finding and Definitive Tests).

In the definitive test, a minimum of 20 organisms is required for the control and each concentration or dilution of the material to be tested. (They can be divided in two or more test containers.) However, use of additional organisms and replication of treatments are desirable, but "loading" must be considered. Replicates, if used, should have no water connection between the replicate test containers. Stratified randomization (random assignment of one test container for each treatment in a row, followed by random assignment of a second test container for each treatment in another or extended row) or total randomization of the treatments is recommended.

The test animals should be distributed impartially to test containers by adding no more than 10 percent to each container, repeating the process until the desired number of test animals is reached in each test container. Animals can be assigned alternatively either by total randomization or by stratified randomization (random assignment of one animal to each test container, random assignment of a second animal to each test container, etc.).

Controls for every test must duplicate the salt water, conditions, and animals (species and size) used in containers with test material. Test results are unacceptable if mortality of control animals exceeds 10 percent.

Toxicant Delivery System--

Flowing sea water tests are preferable to static tests because test solutions are renewed continually, assuring a steady concentration of the toxicant. However, these tests require metering pumps or other devices to deliver the toxicant or test material into the sea water flowing through the test aquaria.

Most toxicant delivery systems have been designed to test toxicants and solvents in fresh water, and may not be applicable in studies of all wastes. Many materials proposed for disposal at sea are not homogenous mixtures; therefore, innovative toxicant delivery systems are required to introduce representative samples of the materials into test containers. Stirring may be required to maintain suspended solids in nonhomogenous dump material.

Many toxicant delivery systems have been described and used in various types of bioassays (Sprague, 1969; Freeman, 1971; Bengtsson, 1972; Cline and Post, 1972; Granmo and Kollberg, 1972; Lowe et al., 1971 and 1972; Lichatowich et al., 1973; Abram, 1973). The proportional diluter (Mount and Brungs, 1967) has probably been used routinely (in fresh water) more than any other system. A small chamber to mix toxicant-bearing water and dilution water should be placed between the diluter and the test containers for each concentration. If duplicate test containers are used, separate delivery tubes can be run from this mixing chamber to each duplicate. Alterations in the design of the proportional diluter have been found useful (Esvelt and Conners, 1971; McAllister, Mauch, and Mayer, 1972; Benoit and Pulglisi, 1973; Schimmel, Hansen and Forester, 1974).

The rate for which water flows through the test containers must be at least five tank-water volumes per 24 hours. It often is desirable to construct a toxicant delivery system that provides 10 or more volumes of tank water per 24 hours. Some systems may provide a continuous flow of sea water. The rate of flow should not vary by more than 10 percent from any test container or for any time period within a given test.

The calibration of the toxicant delivery system should be checked carefully before, during, and after each test. The volume of stock solution and dilution water used in each portion of the toxicant delivery system and the flow rate through each test container must be determined and operation of the toxicant delivery systems checked daily during the test.

Temperature--

Test water temperatures should be the highest average monthly temperature at the discharge site. Suggested water temperatures in Table 1-L represent maximum for surface waters in the coastal regions.

Test water temperature should be maintained within 1°C of average maximum monthly temperature at the dump site or temperature listed in Table 1-L (unless seasonal bioassays are performed). This may be accomplished by preheating the sea water before it enters the test containers, by immersing the test containers in a constant temperature water bath, or by a combination of these methods.

TABLE 1-L. MAXIMUM SEA WATER TEST TEMPERATURES FOR VERTEBRATES AND INVERTEBRATES*

Region	Temperature
I	20°C
II† and III	25°C
IV, VI and IX	30°C
X	15°C

*Temperature in this table should be revised to the highest average monthly temperature of oceanic surface waters at dump sites in each region.

†Puerto Rico and Virgin Islands are in Region II administratively but should use temperatures suggested for Region IV.

Salinity--

The salinity of test water should be that of the dump site if: (a) dump site water is used or (b) artificial sea water is prepared. The salinity of any other natural sea water should be ≥ 15 °/oo.

Loading--

Excessive weight (grams/liter) of organisms in a test container may adversely affect results of test. Therefore, the loading must be limited so that:

1. the concentration of dissolved oxygen in the control container does not fall below 60% saturation;

2. the concentration of metabolic products does not become too high; specifically, the concentration of non-ionized ammonia does not exceed 20 μg/ℓ;

3. the concentration of toxicant is not lowered by more than 20% because of uptake by the test organisms; and

4. the organisms are not stressed by overcrowding.

Loading in the test containers must not exceed 2 g/ℓ/day for species listed under Section 4, "Test Organisms." Lower loadings must be used when necessary to meet the four criteria listed above.

Range-Finding and Definitive Tests--

Time and effort may be saved by "range-finding," static tests using a few animals and a wide range of concentrations, as a preliminary to "definitive" flow-through tests which will be used to calculate the final LC50 or EC50. (See Standard Methods, 14th Edition, 1975 for details.) For example, waste concentrations of 10, 1, 0.1, and 0.01% might be tested first by volume and with two or three animals in each concentration for a period of 24 hours. Definitive test concentrations should then fall between the highest concentration at which all animals survive and the lowest concentration at which all or most animals die.

Observations--

At a minimum, the number of dead or affected animals must be recorded at 24-hour intervals throughout the test. More observations are often desirable, especially in the beginning stage of the test. Dead animals must be removed immediately after observed and their deaths recorded.

Death is the adverse effect most often used to study acute toxicity with aquatic animals. Criteria for death are no movements, especially no opercular movement in fish, and no reaction to gentle prodding. Because death is not easily determined for some invertebrates, an EC50 (effective concentration to 50% of test animals) is often measured rather than an LC50 (lethal concentration to 50% of test animals). The effect generally used for determining an EC50 with invertebrates is immobilization, which is defined as the inability to move except for minor activity of appendages, or loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. General observations on such criteria as erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucous production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism should be reported.

Calculations and Reporting

At the end of the test period, the bioassays are terminated and the LC50 or EC50 values are determined.

Calculations--

An LC50 is the concentration expected to result in 50 percent mortality of the experimental animals and an EC50 the concentration expected to affect 50% of the experimental animals. Either value may be interpolated from percentages of animals dying or affected at two or more concentrations. In interpolating LC50 or EC50, plot data on logarithmic-probability graph paper, placing concentrations on the logarithmic axis and percentage of dead or affected animals on the probability axis. A line is drawn between all data points. The concentration at which the line crosses the 50% mortality or effect line is the LC50 or EC50 value. In fitting the line, points nearest the 50% effect level should be given more weight. Ideally, data should consist of enough intermediate (between 0 and 100%) effects to determine confidence limits by statistical tests (such as probit analysis).

If 50% of the test animals are not affected by the highest concentration, the percentage affected at each concentration must be reported.

Reports--

The final report should include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source, date, and time of collection, composition, known physical and chemical properties, and variability of the material tested;

3. the source of the sea water, date prepared, and method of preparation;

4. detailed information about the test animals, including name, standard length of fishes, carapace width of crabs, total length of shrimp, weight, source, history, and acclimation procedure;

5. a description of the experimental design, the test containers, the volume of test solution, the number of organisms per concentration, and the loading (water flow to each tank);

6. definitions of the criteria used to determine the effect and a summary of general observations on other effects or symptoms;

7. percentage of control organisms that died or were affected in each test container;

8. the 24-, 48-, and 96-hour LC50 or EC50 values;

9. methods used for the results of all dissolved oxygen, pH, and temperature measurements; and

10. any other relevant information.

REFERENCES

Abram, F.S.H. 1973. Apparatus for Control of Poison Concentration in Toxicity Studies With Fish. Water Res. (Oxford) 7(12):1875-1879.

American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater, 14th Edition. Am. Pub. Health Assoc., Wash., DC. 874 p.

Bengtsson, B.E. 1972. A Simple Principle for Dosing Apparatus in Aquatic Systems. Arch. Hydrobiol. (Stuttgart) 70(3):413-415.

Benoit, D.A. and F.A. Puglisi. 1973. A Simplified Flow-splitting Chamber and Siphon for Proportional Diluters. Water Res. (Oxford) 7(12):1915-1916.

Cline, T.F., and G. Post. 1972. Therapy for Trout Eggs Infected With Saprolegnia. Prog. Fish-Cult. 34(3):148-151.

Esvelt, L.A., and J.D. Conners. 1971. Continuous-flow Fish Bioassay Apparatus for Municipal and Industrial Effluents. In: L.A. Esvelt, W.J. Kaufman, and R.E. Selleck. Toxicity Removal from Municipal Wastewaters. Volume IV of "A Study of Toxicity and Biostimulation in San Francisco Bay-Delta Waters." Sanitary Engineering Research Laboratory, Univ. California, Berkeley. pp. 155-182.

Freeman, R.A. 1971. A Constant Flow Delivery Device for Chronic Bioassay. Trans. Am. Fish Soc. 100(1):135-136.

Granmo, A., and S.C. Kollberg. 1972. A New Simple Water Flow System for Accurate Continuous Flow Tests. Water Res. 6(9):1597-1599.

Lichatowich, J.A., P.W. O'Keefe, J.A. Strand, and W.L. Templeton. 1973. Development of Methodology and Apparatus for the Bioassay of Oil. In: Proceedings of Joint Conference on Prevention and Control of Oil Spills. American Petroleum Institute, U.S. Environmental Protection Agency, and U.S. Coast Guard, Washington, DC. pp. 659-666.

Lowe, J.I., P.D. Wilson, A.J. Rick, and A.J. Wilson, Jr. 1971. Chronic Exposure of Oysters to DDT, Toxaphene and Parathion. Proc. Natl. Shellfish Assoc. 61:71-79

Lowe, J.I., P.R. Parrish, J.M. Patrick, Jr., and J. Forester. 1972. Effects of the Polychlorinated Biphenyl Aroclor^{R} 1254 on the American Oyster, Crassostrea virginica. Mar. Biol. (Berl.) 17:209-214.

McAllister, W.A., Jr. W.L. Mauch, and F.L. Mayer, Jr. 1972. A Simplified Device for Metering Chemicals in Intermittent-flow Bioassays. Trans. Am. Fish. Soc. 101(3):555-557.

Mount, D.I., and W.A. Brungs. 1967. A Simplified Dosing Apparatus for Fish Toxicological Studies. Water Res. (Oxford) 1(1):21-29.

Schimmel, S.C., D.J. Hansen, and J. Forester. 1974. Effects of Aroclor 1254 on Laboratory-reared Embryos and Fry of Sheepshead Minnows (Cyprinodon variegatus). Trans. Am. Fish. Soc. 103(3):582-586.

Sprague, J.B. 1969. Review Paper: Measurement of Pollution Toxicity to Fish. 1. Bioassay Methods for Acute Toxicity. Water Res. 3(11):793-821.

M. LABORATORY CULTURE OF SHEEPSHEAD MINNOWS (*CYPRINODON VARIEGATUS*)

D. J. Hansen

Sheepshead minnows can be readily cultured in the laboratory in aquaria with under-substrate filters or in aquaria receiving flowing salt water. The following discussion presents culture techniques used successfully at the U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida 32561.

Spawning Method Using Human Chorionic Gonadotrophic Hormone (HCG)

Although sheepshead minnows spawn naturally in the laboratory, in some instances it is desirable to obtain large numbers of eggs on one particular day. To do this, adult fish $\geq$27 mm standard length should be acclimated for at least two weeks in $\geq$15 ^{o}/oo salinity water at 30°C. Conditions during acclimation should not vary from those recommended by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). Photoperiod should consist of 12 hours of light and 12 hours dark. Fish should be fed *ad libitum* on frozen adult brine shrimp supplemented with dry food. After acclimation each adult female should be injected intraperitoneally with 50 International Units (IU) of HCG to enhance egg production. The next day all females should again be injected with 50 IU of HCG. Three days after the first injection, manually strip, or dissect, eggs from females and deposit them in 25-50 ml salt water of acclimation conditions. Remove testes from five or more males and macerate in a few ml of sea water to free sperm. Mix sperm with eggs in a beaker and place in 30°C water bath for one hour. Embryos then are placed in egg chambers for a life-cycle toxicity test using sheepshead minnows, or placed in suitable hatching chambers.

The advantage of this procedure is that tests can be planned to assure availability of sufficient embryos for life-cycle tests or sufficient juveniles for acute static or flow-through tests after 2 weeks acclimation. However, because females are usually killed or their normal egg production patterns disrupted, this method should be used only occasionally when surplus females are available. If this procedure is followed, the number of eggs produced per female usually averages between 100 and 200, depending on size of females. Fertilization success should be >90 percent.

Spawning Method Using Natural Spawning

It is sometimes desirable to obtain a continuous supply of sheepshead minnows for toxicity tests by using natural reproduction of laboratory-held fish. To use this method, adult fish $\geq$27 mm standard length should be acclimated for at least two weeks in $\geq$15 ^{o}/oo salinity water at 30°C. Conditions during acclimation should be identical to those described for spawning using HCG. Fish are placed in spawning chambers spacious enough to prevent deaths due to aggressive territoriality by males and cannibalism of eggs. Spawning chambers as described in the section, "Entire Life-Cycle Toxicity Test Using Sheepshead Minnows," have proven successful at ERL, Gulf Breeze.

The number of spawning chambers and fish to be spawned should be based on the requirements for providing sufficient embryos. A pair of fish will generally produce an average 10 to 30 eggs each day while held in spawning chambers in water of ≥15 °/oo salinity and temperature of 30°C. The number of fish successfully held in our spawning chambers ranged from one pair to two males and five females.

Rearing Methods for Embryo, Fry, and Juvenile Sheepshead Minnows

Two hatching techniques that have proven most successful require placement of embryos in (1) flowing salt water aquaria chambers as described in the life-cycle toxicity test method or (2) in static salt water in separatory funnels which are supplied air through a 2.5-mm ID glass tube that extends to the bottom of the funnel. Air flow to the tube should be only fast enough to keep embryos and hatched fish suspended in the water column. Hatching time required for sheepshead minnows depends on temperature of water, and survival depends on temperature and salinity (Schimmel and Hansen, 1974). Embryos hatch most rapidly and their survival is optimum in water ≥15 °/oo salinity and 30°C temperature. Unfortunately, we have yet to find an artificial sea salt that can be used in rearing embryos.

After embryos hatch, the fish are removed from embryo and fry chambers, or separatory funnels, and placed in aquaria for acclimation for toxicity tests. Salinity and temperature should be adjusted for acclimation as suggested for acute toxicity test methods. Fish are fed live brine shrimp nauplii.

REFERENCES

Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. EPA-660/3-75-009. U.S. Environmental Protection Agency, Cincinnati, OH.

Schimmel, Steven C., and David J. Hansen. 1974. Sheepshead Minnow (*Cyprinodon variegatus*): An Estuarine Fish Suitable for Chronic (Entire Life-cycle) Bioassays. Proc. 28 Annu. Conf. Southeast. Assoc. Game Fish Comm. pp. 392-398.

N. LIFE-CYCLE TOXICITY TEST USING SHEEPSHEAD MINNOWS (*CYPRINODON VARIEGATUS*)

D.J. Hansen, P.R. Parrish, S.C. Schimmel, and L.R. Goodman.

PURPOSE AND LIMITATIONS

This procedure provides a method to determine the effect of continuous exposure of a toxic material on sheepshead minnow embryos and fry: their survival and growth to adulthood, and spawning success. Spawning success is measured by the ability of fish to spawn naturally, number of eggs spawned, fertilization success, and survival of embryos and fry. The experiment requires from 4 to 6 months.

The primary advantage of this test is that results, when compared with those of acute tests with this species, can be used to calculate an application factor (Mount & Stephan, 1967). This factor, used to assess relative chronic hazards of materials, is important in establishing water quality criteria (Eaton, 1973; Hansen and Parrish, 1977).

This test has several limitations and should not be considered valid in assessing toxicity of all materials. Sheepshead minnows can tolerate low dissolved oxygen and wide ranges of temperature and salinity. Therefore, toxicity tests using this fish may underestimate the toxicity of materials that alter these environmental conditions. Materials tested should mix well with water. Insoluble or highly turbid materials mix poorly, and their toxicity may be under- or overestimated.

Physical Systems

a. Test Water

1. The source of test water should be (1) from the dump site or (2) natural seawater with salinity ≥ 15 $^{o}/oo$.

2. Sea water must be filtered to remove particles 15μ and larger, but filtration should not affect the chemical composition of the natural sea water. Filtration must remove planktonic larvae which prey upon eggs, fry, and juvenile fish.

3. Any sea water source proposed must be analyzed for possible pollutants (e.g., pesticides, PCB's, heavy metals, and the material to be tested).

b. Dosing Apparatus

A number of apparatus are acceptable for this bioassay. For example, use the device described by Mount and Brungs (1967) or Hansen et al. (1971) for substances not requiring a solvent. However, if a solvent is required, use the device described by Hansen et al. (1974) or Schimmel et al. (1974).

c. Toxicant Mixing

A mixing chamber is necessary to assure adequate mixing of the test material. Aeration should not be used for mixing. Mixing is extremely important because if materials are not adequately mixed with water, toxicity cannot be properly assessed. Improper mixing can either expose the animal to too much or too little of the material, causing toxicity to be over- or underestimated. Therefore, scientific judgment should be used for designing and selecting appropriate dosing apparatus and mixing systems.

d. Duplicates

True duplicates are used for each concentration in all tests (no water connection between aquaria). Aquaria are located by random selection.

e. Aquaria

Glass aquaria, 45 x 90 x 26 cm high and with a water depth of 19 cm, have been used successfully.

f. Embryo and Fry Chambers

1. Embryo and fry chambers must allow for adequate exchange of water and insure that the proper quantity of material enters the chambers. Chambers can be constructed from Petri dishes to which 40-mesh nylon or a stainless steel screen is glued (Schimmel et al, 1974). The Petri dish chambers are placed in aquaria that have a self-starting siphon. Water from the dosing apparatus fills the aquaria to the level required to start the siphon. Water then drains from the aquaria, flowing in and out of the embryo and fry chambers. Chambers can also be constructed from 5-cm OD round glass or beakers without bottoms. The bottoms are replaced with 40-mesh stainless steel or nylon screening. Chambers are suspended in the test water on an oscillating rocker arm apparatus that is driven by a 1-5 rpm electric motor (Mount, 1968). These chambers must be brushed daily to prevent clogging.

2. Embryo and fry chambers must be designed so water can be drained to 1 cm, or the fry removed for observations and measurement.

3. Embryo and fry chambers may be supplied test water by: (1) separate delivery tubes from the mixing chamber, (2) self-starting siphons in the aquaria, or (3) an oscillating rocker arm apparatus.

g. Flow Rate

1. Flow rates to each duplicate aquarium must: (1) provide 90% replacement in 8 to 12 hours (Sprague, 1969); (2) maintain dissolved oxygen 60% saturation; and (3) maintain the toxicant concentration.

2. The test system is equipped with an alarm system to insure continuation of water flow, toxicant flow, and temperature regulation.

h. Photoperiod

A 12-hour light/12-hour dark cycle is maintained throughout the test. It may be desirable to control lights by a timing switch (Drummond and Dawson, 1970). Lighting above each replicate must be balanced.

i. Temperature

Test temperature is maintained at 30°C (+1°C) by either (1) preheating the diluted water to the prescribed temperature, and/or (2) placing test aquaria in a temperature-controlled water bath. A continuous record of temperature of test water must be kept.

j. Cleaning

All aquaria are cleaned whenever organic material builds up. Aquaria are brushed down and siphoned to remove accumulated material. Fish can be left in the aquaria, but the end of the siphon is covered with screen. Care should be exercised in cleaning to prevent loss of or damage to the fry, juveniles, or adults. Embryo and fry chambers may have to be replaced or cleaned frequently if screens clog or organic material collects. When a chamber is cleaned, it can be reused only in the aquarium from which it was removed. Special care is required to prevent injury to fry.

k. Spawning Chambers

Chambers are constructed of either glass or #316 stainless steel (Hansen et al., 1977). Chambers 20 x 35 x 22 cm high have been used successfully. A 2.0-mm mesh screen is attached 1 cm above the bottom of a removable "drawer" to facilitate passage of eggs, thereby reducing cannibalism of eggs by parents. A "drawer" of 0.5-mm mesh nylon or 316 stainless steel screen will catch eggs falling through the screen to the bottom of spawning chambers. Fish in the test aquarium outside the spawning chamber must be prevented from eating the eggs. This is accomplished by a partition or by a drawer constructed so that fish have no access to eggs.

l. Disturbance

Fish are shielded from excessive outside disturbances. Tanks will eliminate outside light sources that interfere with the photoperiod. Preferably, an opaque curtain surrounds the entire test apparatus.

m. Concentrations

1. A minimum of five concentrations of toxicant and a control, all duplicated, are utilized in all chronic tests. When a solvent is used, the control contains the solvent.

2. One concentration selected must adversely affect a life stage of the sheepshead minnow and one concentration must not affect any life stage. Concentrations selected for chronic toxicity tests are based on results of acute flow-through toxicity tests. Selection of test concentrations is difficult because chronic effects on survival, growth, or reproduction of sheepshead minnows can occur at concentrations that range from 0.5 to 0.0001 of the 96-hour LC50. The accuracy of the selection process can be improved by conducting preliminary tests such as: (a) acute 96-hour flow-through tests using different life stages (e.g., fry, juvenile, and adults), (b) acute test to determine incipient LC50 (Sprague, 1969), or (c) embryo-fry tests (Schimmel & Hansen, 1974). The highest concentration in the life-cycle test, in most instances, should be the lowest concentration affecting survival or growth in preliminary tests.

3. Chemical analyses are required to interpret results of this complex bioassay: Water and a minimum of 10 or more fish should be analyzed for each aquarium, but preferably water and muscle and gametes of fish in each life cycle should be chemically analyzed weekly.

n. Acute Tests

Acute flow-through toxicity tests using juvenile fish must be conducted. Consult section on suggested acute flow-through bioassay methods.

Biological Systems

a. Source of Adult Fish

Adult fish are obtained from the one source, either from wild populations or suitable culture laboratories; wild stocks may be preferable. They are held in flowing 30°C sea water of ≥ 15 $^{o}/oo$ salinity for at least two weeks before the eggs are removed. Neither fish nor eggs should contain excessive contaminants nor exhibit excessive mortality; fish should demonstrate normal behavior. (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975.)

b. Eggs from Adult Fish

To obtain a sufficient number of eggs for a chronic exposure, two methods may be employed: (1) natural spawning from laboratory stocks; and (2) artificial inducement, in which egg production is stimulated by injection of human gonadotrophic hormone. Eggs are removed from females and are fertilized in salt water with sperm excised from males (Schimmel et al., 1974). Consult section on culturing sheepshead minnows.

c. Test Implementation

The test begins after 50 microscopically confirmed embryos are placed in two or more embryo and fry chambers in each duplicate aquarium. Survival of embryos and fry (which constitute the parental stock) are observed and recorded daily. Occurrence of abnormalities in embryos and fry, and their frequency, are important indicators of teratogenicity. Also, signs of poisoning should be observed and recorded as indicators of mode of action of the toxicant. Effects of toxicants on behavior of this fish can be as significant as, or more significant than, effects on survival, growth, or reproduction.

d. Food

1. Fry are fed equal portions of live brine shrimp nauplii two or more times daily for about two weeks. (Do not use frozen nauplii.)

2. Juveniles and adults can be fed twice daily on equal portions of dry food (e.g. BiOrell^R or Tetramin^R) supplemented with frozen adult brine shrimp. Each batch of food should be checked for pesticides (DDT, dieldrin, endrin, etc.) and metals. In addition, chemical analysis should also include chemicals in the material to be tested.

e. Disease

If disease occurs, a test preferably is terminated and started again. If diseased animals are treated, they should be handled according to their nature. Each aquarium is treated identically even though disease is not evident in all aquaria. Treatments should be kept to the minimum and recorded as to type, amount of medication, and frequency.

f. Measurements

Fish of the parental generation are measured in mm standard lengths at four weeks before removal of extra fish. Therefore, measurements are taken at four-week intervals and at adult termination. Juvenile (F_1) fish are to be measured at week four (termination of test). Techniques suggested for measuring fish include a photographic method outlined by McKim and Benoit (1971) and direct measurement at termination.

g. Thinning

At day 28, juvenile fish are randomly reduced to 25 fish per duplicate aquaria, providing enough fish for at least two spawning groups of three adult females and two males in each duplicate aquarium for observations on effects on spawning.

h. Spawning

When mature adults begin courtship (indicated by sexual dimorphism, territoriality, and aggressive behavior by the male), and attain a minimum standard length of 27 mm, three females and two males are placed in individual spawning chambers in the test aquaria. Fish from each spawning group are left in chambers for a minimum 14 days. All (possible) fish in the 2:3 ratios in each aquarium are spawned and extra, unspawned fish from each duplicate aquarium are combined whenever feasible to form additional 2:3 spawning groups. Adult deaths during spawning should be noted; dead animals are removed, measured, but not replaced. At termination of each spawning group, lengths and weights of individual fish are measured.

i. Egg Removal

Records of egg numbers and egg fertility are maintained daily. All eggs must be removed daily, examined for fertility, reserved for survival studies or residue analyses, or discarded. Eggs are removed at a fixed time each day so spawning activity is not disturbed unnecessarily.

j. Egg Incubation

1. Fifty embryos are collected and incubated from adults in each aquarium. It may be desirable to obtain 25 from one day's spawning by each of two spawning groups. If spawns are small, the 50 embryos can be collected over an extended period.

2. If no spawning occurs in the highest concentration, embryos are transferred from control spawns and incubated in the highest concentration to gain additional information.

3. Groups of 50 embryos are divided into two-egg cups. Survival of embryos, time required to hatch, hatching success, and survival of fry for four weeks are determined and recorded.

4. Additional groups of 50 embryos from fish from contaminated aquaria should be rinsed with control water and then placed in control aquaria to determine if the eggs contain chemicals toxic to embryos or fry.

k. Embryo, Fry, and Juveniles (the F_1 generation)

Survival of embryos and fry is recorded daily for four weeks. Daily observations are made of embryos and fry; mortalities, development of abnormalities, and signs of poisoning are recorded. Length and weight of juvenile fish is measured at test termination (day 28); weight may represent the total for all fish in each fry chamber. Fish may be saved for chemical analyses.

l. Termination of Adults

1. In many chronic procedures utilizing other fishes, tests are terminated when no spawning activity occurs for a two-week interval. Tests using the sheepshead minnow, however, should terminate after a spawning is observed for two weeks because this fish spawns readily and almost daily unless immature or affected by a pollutant. The effect of the toxicant on each group spawning in the 2:3 ratio is tested and each group then terminated. Final termination follows tests of all spawning groups.

2. Adult fish are weighed, measured for standard length, sexed, and retained for residue analysis.

m. Additional Tests

Certain materials may contain substances that require additional tests to determine physiological or pathological effects on one or more life stage of the sheepshead minnow.

Statistical Analyses

The LC50's and 95% confidence limits are calculated on data from acute tests by probit analysis. Data from life-cycle bioassays are analyzed by analyses of variance, or chi-square tests. Post hoc tests are conducted on treatment means using the Newman-Keuls range test.

REFERENCES

Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975. Methods for Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. EPA-660/3-75-009. U.S. Environmental Protection Agency, Cincinnati, OH.

Drummond, Robert A., and Walter F. Dawson. 1970. An Inexpensive Method for Simulating Diel Patterns of Lighting in the Laboratory. Trans. Amer. Fish Soc. 99(2):434-435.

Eaton, J.G. 1973. Recent Developments in the Use of Laboratory Bioassays to Determine "Safe" Levels of Toxicants for Fish. G.E. Glass, Ed. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich. pp. 107-115.

Hansen, D.J., and P.R. Parrish. 1977. Suitability of Sheepshead Minnows (*Cyprinodon variegatus*) for Life-cycle Toxicity Tests. Aquatic Toxicology and Hazard Evaluation. ASTM STP 634. F.L. Mayer and J.L. Hamelink, Eds. American Society for Testing and Materials, pp. 117-126.

Hansen, D.J., S.C. Schimmel, and J. Forester. 1974. Aroclor^{R} 1254 in Eggs of Sheepshead Minnows (*Cyprinodon variegatus*). Effect of Fertilization Success and Survival of Embryos and Fry. Proc. 27th Ann. Conf. Southeast. Assoc. Game Fish Comm. Oct. 1973. Hot Springs, Arkansas: 420-426.

Hansen, D.J., S.C. Schimmel, and J. Forrester. 1977. Endrin: Effects on the Entire Life-cycle of a Salt Water Fish. J. Toxicol. Environ. Health 3:721-733.

Hansen, D.J., P.R. Parrish, J.I. Lowe, A.J. Wilson, Jr., and P.D. Wilson. 1971. Chronic Toxicity, Uptake, and Retention of Aroclor^{R} 1254 in Two Estuarine Fishes. Bull. Environ. Contam. Toxicol. 6:113-119.

McKim, J.M., and D.A. Benoit. 1971. Effect of Long-term Exposures to Copper on Survival, Growth, and Reproduction of Brook Trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 28(5):655-662.

Mount, Donald I. 1968. Chronic Toxicity of Copper to Fathead Minnows (*Pimephales promelus*, Rafinesque). Water Research 2:21-29.

Mount, D.I., and C.E. Stephan. 1967. A Method for Establishing Acceptable Toxicant Limits for Fish-malathion and the Butoxyethanol Ester of 2,4-D. Trans. Amer. Fish. Soc. 96(2):185-193.

Mount, Donald I. and William Brungs. 1967. A Simplified Dosing Apparatus for Fish Toxicology Studies. Water Research 2:21-29.

Schimmel, S.C. and D.J. Hansen. 1974. Effects of Aroclor^R 1254 on the Embryo and Fry of Sheepshead Minnows. Trans. Amer. Fish. Soc. 103(3): 522-586.

Sprague, J.B. 1969. Review Paper: Measurement of Pollution Toxicity to Fish. 1. Bioassay Methods for Acute Toxicity. Water Research 3(11): 793-821.

O. FISH BRAIN ACETYLCHOLINESTERASE INHIBITION ASSAY

D. L. Coppage

INTRODUCTION

This procedure provides a method for determining the effect of materials to be dumped in the ocean on acetylcholinesterase (AChE) in fish brains. This test is appropriate for nerve poisons which disrupt nerve impulse transmission by inhibiting AChE, the enzyme that modulates levels of the neurotransmitter acetylcholine (Koelle, 1963; Karczmar, 1970). This procedure is not necessary for materials that contain no AChE inhibiting poisons.

It has been shown that brain-AChE of fishes is inhibited by in vivo exposure to organophosphate and carbamate pesticides under laboratory conditions (Weiss, 1958, 1961; Carter, 1971; Coppage, 1972). Furthermore, environmental water pollution by these pesticides has been monitored by measuring AChE activity in fish brains (Williams and Sova, 1966; Holland et al., 1967; Coppage and Duke, 1971). Coppage (1971) defined the conditions necessary for obtaining reliable and reproducible data in the laboratory AChE assays and reported in vitro effects of four pesticides on AChE activity in brains of sheepshead minnows (Cyprinodon variegatus). Coppage and Matthews (1974) further refined assay techniques and reported acute effects of in vivo exposure to organophosphate pesticides on cholinesterases of four estuarine fishes and a shrimp.

Recommended Procedure for Exposing Animals

Fish should be exposed to the material as recommended in the definitive test of the continuous-flow method for acute toxicity tests, using fish and macroinvertebrates as described in this manual. Fish to be assayed for AChE should be from control aquaria and, if possible, from three contaminated aquaria in which some fish have died. Live control fish should be divided into three groups of three to six fish for assay. Three to six fish from the contaminated aquaria should also be assayed by the method described below.

Recommended Procedure for AChE Assay

Preparation of Fish Brains (3 to 6 brains are pooled for each sample)--

1. Weigh 5 cm square of aluminum foil in following manner: pick up and place foil on balance pan with forceps. (Fingers can leave enough moisture to cause weight error at this low weight.) Weigh, then leave on pan at full rest.

2. Place another larger piece of foil in dissecting area.

3. Kill fish by placing them in a clean beaker containing acetone for about 3 minutes.

4. Pour fish into clean sink or pan and scale heads under running water with scalpel. (Check to ascertain that all dissecting equipment has been cleaned and rinsed with acetone. As heads are scaled, place fish in another beaker containing acetone.)

5. Pour fish into clean sink or pan, and then blot fish dry on paper.

6. Clip top of skull from the brain with scissors.

7. After all skulls have been clipped, remove brains by pulling off bone flap with forceps and digging bone and flesh away from spinal cord with probe if necessary. Cut spinal cord about 2 mm behind brain.

8. Strip brain from optic nerves, and place on larger piece of foil.

9. After removal, transfer brains with forceps to the preweighed foil on the balance pan and determine weight in milligrams. Divide weight by five.

10. Transfer weighed brains to nylon cup (see next section) and add about 4 ml of distilled water.

11. Homogenize for 1 minute, then pour into graduated cylinder. Rinse cup several times with distilled water and pour into cylinder.

12. Add distilled water to cylinder until total volume (in ml) equals the amount found by dividing the brain weight by five. Pour this into beaker to gently mix. Assay within 30 minutes after preparation.

Assay for AchE--

AChE activity should be determined by using an automated recording pH stat to measure normal and *in vivo*-inhibited brain AChE. The following procedure applies: mix 2 ml of diluted brain homogenate with 2 ml of 0.03 M acetylcholine iodide in distilled water; titrate the liberated acetic acid with carbonate-free 0.01 N NaOH; carry out the reaction at pH 7 and 22°C while passing nitrogen over the liquid to prevent adsorption of atmospheric carbon dioxide. Calculate the micromoles of substrate hydrolyzed per unit of time from the number of micromoles of NaOH required to neutralize the liberated acetic acid per unit of time; express AChE activity as micromoles of ACh hydrolyzed per hour per mg brain tissue in reaction vessel.

Calculations and Reporting

Assay results of exposed and control fishes are compared, and percentages of normal brain AChE activity of exposed fish are reported. Results should be subjected to statistical analysis (Student's t-test, for example) to determine statistical validity. Original control fish may be divided into groups of five and brains pooled for each group of five to obtain samples for normal AChE and statistical comparisons with exposed fish replicates.

Reports

Any deviation from this method must be noted in all reports of results. A report of the results of a test must include:

1. name of method, author, laboratory, and date tests were conducted;

2. a detailed description of the material tested, including its source, date and time of collection, composition, known physical and chemical properties, and variability of the material tested;

3. the source of the salt water, its date and method of preparation;

4. detailed information about the test animals, including name, standard length, weight, age, source, history, and acclimation procedure used;

5. a description of the experimental design, the test containers, the volume of test solution, the number of organisms per concentration, and the loading;

6. period of exposure and number of animals dead at end of exposure;

7. percentage of control organisms that died or were affected during the test;

8. methods used for and the results of all test material, dissolved oxygen, pH, and temperature measurements; and

9. any other relevant information.

REFERENCES

Carter, F.L. 1971. *In vivo* Studies of Brain Acetylcholinesterase Inhibition by Organophosphate and Carbamate Insecticides in Fish. Unpublished Ph.D. dissertation, Louisiana State Univ., Baton Rouge, Louisiana.

Coppage, D.L. 1971. Characterization of Fish Brain Acetylcholinesterase With an Automated pH Stat for Inhibition Studies. Bull. Environ. Contam. Toxicol. 6(4):304-310.

Coppage, D.L. 1972. Organophosphate Pesticides: Specific Level of Brain AChE Inhibition Related to Death in Sheepshead Minnows. Trans. Am. Fish. Soc. 101(3):534-536.

Coppage, D.L., and T.W. Duke. 1971. Effects of Pesticides in Estuaries along the Gulf and Southeast Atlantic Coasts. In: Proceedings of the 2nd Gulf Coast Conference on Mosquito Suppression and Wildlife Management, pp. 24-31. (C.H. Schmidt, Ed.) National Mosquito Control-Fish and Wildlife Management Coordinating Committee, Washington, D.C.

Coppage, D.L., and E. Matthews. 1974. Short-term Effects of Organophosphate Pesticides on Cholinesterase of Estuarine Fishes and Pink Shrimp. Bull. Environ. Contam. Toxicol. 11(5):483-488.

Holland, H.T., D.L. Coppage, and P.A. Butler. 1967. Use of Fish Brain Acetylcholinesterase to Monitor Pollution by Organophosphorus Pesticides. Bull. Environ. Contam. Toxicol. 2(3):156-162.

Karczmar, A.G. (Ed.). 1970. Anticholinesterase Agents. Pergamon Press, New York.

Koelle, G.B. (Ed.). 1963. Cholinesterases and Anticholinesterase Agents. Springer-Verlag, Berlin.

Weiss, C.M. 1958. The Determination of Cholinesterase in the Brain Tissue of Three Species of Fresh Water Fish and Its Inactivation *in vivo*. Ecology 39:194-199.

Weiss, C.M. 1961. Physiological Effect of Organic Phosphorus Insecticides On Several Species of Fish. Trans. Am. Fish. Soc. 90:143-152.

Williams, A.K. and R.C. Sova. 1966. Acetylcholinesterase Levels in Brains of Fishes From Polluted Waters. Bull. Environ. Contam. Toxicol. 1:198-204.

* U.S. GOVERNMENT PRINTING OFFICE: 1978--740-263/1641 Region No. 4